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More muddle about nuclear weapons

Does the United States know what it is saying on nuclear strategy? Does it appreciate the consequences in Europe? Will it properly represent European interests at the arms control talks beginning 30 November?

The government of the United States lacks a way with words. So much is clear from the declarations of President Reagan and his Secretaries of State and of Defense in the past few weeks about the likely course of nuclear war in Europe. All three members of the Administration have let slip statements on this complicated issue which are dangerously over-simple. Have these high officials of state never learned to qualify simple declarative statements of policy with the conditional clauses that are usually necessary? Whatever the explanation, their gaffes have thrown a cloud over the negotiations due to begin in Geneva at the end of the month between the Soviet Union and the United States on European nuclear weapons. People in Europe, to say the least, are wondering whether officials so unsubtle in their use of language can be trusted fairly to represent the European case at these crucial talks.

The dispute two weeks ago between Mr Alexander Haig and Mr Caspar Weinberger about NATO's plans for demonstrating a nuclear weapon in some future crisis says more about personal relationships within the Administration than about the nuclear defence of Europe. On the Wednesday, Mr Haig was telling the Senate Foreign Relations Committee that NATO's contingency plans include one to "demonstrate to the other side that they are exceeding the limits of toleration". The following day, Mr Weinberger told the Senate Armed Services Committee that there is no such plan — nor "should" there be. Later, the White House announced that both secretaries were telling the truth. So, plainly, they are. What Washington now calls a "demonstrational" nuclear weapon must obviously be an option for NATO to consider if a conventional conflict should break out in Europe. On the face of things, such a device would be preferable to the use of nuclear weapons against targets on the ground. But it is virtually impossible to tell in advance when such a demonstration might be effective. What the Secretaries of State and Defense were talking about was what NATO calls the doctrine of "flexible response". They should have said so.

President Reagan's own slip of the tongue is more serious. In answer to a question from a group of newspaper editors three weeks ago, he said it is conceivable that some future nuclear war might be confined to Europe. Many Soviet strategists share this belief, even though Mr Leonid Brezhnev consistently denies it with gloomy accounts of how any nuclear war would visit destruction on the whole world. Mr Brezhnev is probably nearer the mark than Mr Reagan, for if by evil chance a single nuclear weapon is used in anger, there is no obvious stopping place on the ladder of escalation. Yet the NATO doctrine of flexible response supposes that a conventional incursion across the North German plain would have to be countered, after a few days, by "tactical" nuclear weapons. The hope is that this response would bring the incursion to a halt. If that happened, Mr Reagan would be right.

Yet this calculation is a gamble, and one with long odds. The strategists agree that the conflict would more probably escalate and that the United States would itself be drawn in. Precisely that probability is the foundation for NATO's strategic assumption that nuclear weapons are a peace-keeping device, a deterrent. Moreover, the argument works in both directions. The decision in 1979 that American nuclear weapons in Europe should be modernized by means of Pershing II and cruise missiles was intended by the then United States Administration to strengthen

the linkage between Western Europe and the United States. It is simply wrong of Mr Reagan to fail to point that out when speculating about the possibility that a nuclear war might be confined to Europe. The most serious damage done by his and his colleagues' remarks, however, is that Europeans' willingness to accept the theory of deterrence, by its nature bizarre, has been further undermined. Washington's willingness to talk about nuclear warfare as if it were just an elaboration of conventional warfare suggests that it does not understand what its strategy is for.

What does this imply for the talks beginning on 30 November in Geneva? At first sight, the prospect is not bright. Past experience, however, has shown that there is nothing more educative of the superficial understanding of politicians than negotiations about arms control. Six months from now, President Reagan and his colleagues will be wiser men, perhaps even regretting their delay in sitting down to talk. Moreover, it now appears that the United States has moved a long way to accommodate the anxieties of European governments about nuclear weapons. Last weekend, the Administration let it be known that its opening bid at Geneva will be an offer to dispense with Pershing II and cruise missiles (due to appear in Europe in 1983) if only the Soviet Union will remove its SS20 missiles (together with the older SS4 and SS5 missiles). The Soviet Union will predictably reply that the American offer says nothing about the French and British nuclear weapons. Sooner or later, one side or the other will acknowledge that aircraft cannot be entirely ignored. And the United States will have to decide what to do about Salt II.

Even so, the months ahead are potentially fruitful for the cause of arms control. Indeed, next year will also bring a special assembly of the United Nations on arms control, which could be valuable if states with only marginal interest in the subject can be prevented from making speeches about instant and comprehensive disarmament. There may also be a conference on arms control in Europe within the framework of the Helsinki agreements. It would make sense that the issue of a comprehensive ban on testing nuclear weapons should be given another airing, ideally as part of a Salt III, but the chances of that are slim — too much is at stake for the superpowers readily to agree. The potential beneficiaries of any progress in these directions are several. Many states, in Eastern as well as Western Europe, would welcome the civility and the greater political flexibility that further measures of arms control would bring. Even the superpowers, however, should benefit — President Reagan should be glad if part of his prospective budget deficit melts away, for example.

There are, however, several potential snags. Just as negotiations on arms control are not merely better than no negotiations but are also in themselves a source of reasonableness, arms control negotiations that break down are a source of danger. The United States government has only itself to blame if its self-contradictions of the past few weeks have raised doubts about its seriousness, and its stomach for the tedium that lies ahead. More seriously, the proposal that the opening bid in the Geneva negotiations should abolish all potentially strategic nuclear missiles in Europe is likely, before many months have passed, to boomerang. The American missiles were, after all, intended as a guarantor of American involvement in European defence; will not their absence seem to many a proof of American detachment?



Nobody should be surprised if, some months from now, present anxieties about nuclear weapons based in Europe are outmatched by fears that, without them, one half of Europe or the other will be defenceless.

Finally, there is the problem — strictly, the red herring — of Mr Brezhnev's proposal for an international committee of distinguished scientists to consider the consequences of nuclear war and to tell the world what they are. In the past few months, the Soviet Union has introduced resolutions to this effect in the general assemblies of the World Health Organization and the UN Environmental Programme. By all accounts, a similar proposal will soon be put to the general assembly of the United Nations. In reality, however, there is no shortage of documents spelling out the horrors of nuclear conflict. Most of them agree that the consequences would be appalling. What is needed is not another chilling document but a more constructive attempt to show how nuclear war can realistically be avoided. While the scientific community has much to contribute to such an investigation, it has no special competence to say what is strategically and politically possible. But that, of course, is why the two superpowers are sending delegations to Geneva.

Equity in research

British research councils are saying that universities must also invest in research. But can they? And fairly?

The Medical Research Council has helpfully made public the letter in which it has told British universities that they need not apply for research grants on behalf of department which are inadequately equipped (see page 201). By this means, universities will at least know where they stand. And it is only right that a substantial grant-making agency such as the council, which is in principle well placed to compare the utility of money spent in university departments and in its own establishments, should do what it can to ensure that grants are not wasted because universities cannot give their recipients adequate support. One problem, of which the council must be well aware, is that in the process of saying no to applicants whose universities cannot support them properly, it may be denying good people a run for their money. For who can be sure that the academic departments which universities decide must be cut back will never include among their staffs potentially creative people? By doing what it must — concentrating support in the departments that universities themselves decide to back generously — the council (like other British research councils) is in danger of backing mediocre horses. This is another way of saying what has been clear all along — that the dual-support system (by which universities pay for the overhead cost of academic research, and research councils for the extras) has long since broken down. The question now is what should be done to put things right. To remark that a committee under Sir Alec Merrison has been brooding on the question for the past two years is not a sufficient answer.

The plain truth is that it will be intolerable (but also a dangerous waste of talent) if research councils such as the Medical Research Council think it prudent not to back particular people because the universities concerned have chosen to be mean to the departments in which those people work. The simple solution is obvious but probably unattainable — let the British research councils pay the full cost of the research projects that they back, transferring the cost of the overheads they would then incur from the budget of the University Grants Committee: the universities would kick up too much of a fuss. Another would be to consider grant applications from all university departments, and to use research council money to help ensure that successful applicants are able to migrate to places where research could be carried out effectively. The device now used in the Netherlands (see page 202) by which universities are subsidized under the two separate headings of teaching and research would be even less welcome to British universities — but, on that account, might be an efficient spur to change. Whatever stratagem is used, there is a crying need that

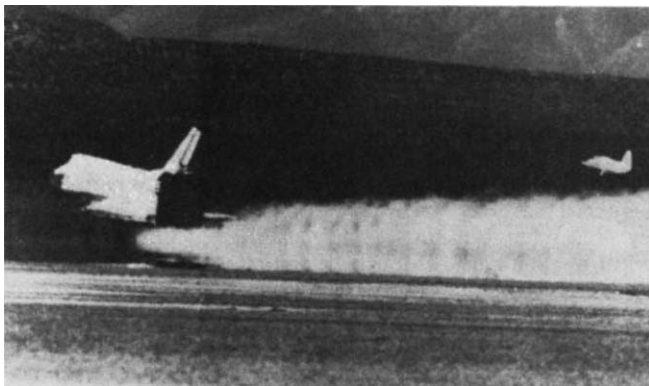
something should change. For to be unable to make research grants to people likely to use them well is not merely inequitable to those concerned but unfair to the rest of us.

Come back, Columbia

The shuttle looks like a success. Might it be a better one if NASA had time to think?

The second test-flight of the shuttle spacecraft was in no sense the near-calamity it has been represented to have been. That one out of three fuel cells should have functioned inefficiently, but that the spacecraft should nevertheless have performed well for more than two days, can just as well be taken as a proof of the good sense of its designers and of the flexibility of the system they have devised. It is not as if the shuttle were simply another kind of aircraft, whose development would no doubt have followed a more conventional course, with a succession of test-flights gradually extending its performance. Instead, it has gone almost in one jump from the drawing-board to full flight. In the steady enlargement of the regimen accessible to manoeuvrable passenger-carrying vehicles, the shuttle is a landmark of an innovation. Two years late though it may be, the US National Aeronautics and Space Administration deserves full credit for making it fly at all.

Two groups of problems nevertheless persist. First, the cost of the remaining development threatens to be an insupportably large fraction of the total space budget, with the result that NASA cannot sustain its modest interest in the less spectacular parts of its research programme even though it has been shielded from the full rigours of the 12 per cent budget cut decreed last month. It



cannot be that this is what the White House intends. And even though the potential military importance of the shuttle has led some to suggest that the Pentagon might shoulder some of the responsibility for it, its importance as an economical means of launching satellites of great commercial importance is, rather, an argument (if one were needed) that budget-balancing by means of across-the-board cuts is no way to conduct government business. But there are also problems with the shuttle itself, last week's success apart. Now, not later, is the time to be sure that solutions adopted ten years ago to technical problems such as the heating of the spacecraft in the upper atmosphere are still the best.

The important need, now, is to get the shuttle right. The United States space administration is understandably anxious not to delay much more. The customers are restive, and the political dangers for an agency that fails to deliver could be serious. But, to many people's surprise, space travel in the modest mould represented by last week's Columbia has come to stay. It will be economically important and, in the long run, a cheerful influence on people's spirits. The space agency will no doubt say that all the technical problems — the engines, the heat shield, the turn-round time and the next rocket stage — are continually being looked at. It would be best if this re-examination could be comprehensive, and public. For then the pressures from would-be customers, at the Pentagon and elsewhere, would be diminished. As would the risk of losing great benefits for want of a little forethought.

US plans help for nuclear industry

Civil power to link with weapons?

Washington

The Reagan Administration has offered the US nuclear industry all of the moral — and some of the economic — support that it wants. But even this may not be enough to restore the industry to health according to the parting words of the Nuclear Safety Oversight Commission (NSOC), an independent advisory body set up by President Carter following the Three Mile Island accident in March 1979.

Three main options face the Administration if it wants to save the civil nuclear industry, according to Governor Bruce Babbitt of Arizona, chairman of NSOC. He presented President Reagan with this opinion when transmitting the commission's final report last month. Babbitt warned, however, that each of the options would require a high political price.

The first option would be to regionalize the nuclear industry and draw it into the public power grids, a form of semi-nationalization already adopted, for example, by the Tennessee Valley Authority. The second would be to bail out the industry directly through government subsidies, although allowing it to remain largely in private hands.

The third and perhaps most controversial option would be to re-establish links between the commercial uses of nuclear power and the military demands for nuclear weapons, using the expansion of the latter to sustain the former. Some critics argue that the Administration's plan to use plutonium extracted from commercial wastes to provide fuel for weapons is already a step in this direction.

This drastic set of choices reflects the serious problems facing the industry. The most popular scapegoats have been the tough environmental regulations and stringent licence review procedures; but no less important has been the reduction in the rate of growth in power demand from 7 to 3 per cent a year over the past decade, and continued public concern about safety (highlighted by the design mistakes found in the Diablo Canyon reactor under construction in California).

The Administration has already taken several steps to assist the nuclear industry. In an attempt to encourage nuclear exports, for example, it is offering countries such as Mexico cut-price enriched uranium, and scientific collaboration in areas such as waste disposal and reactor safety.

Other measures are being taken to speed

up the licensing of new plants. In a policy statement issued last month, Mr Reagan listed the goal of reducing the time needed to license and operate a new nuclear reactor from an average of 12 to 7 years as one of five steps intended to revitalize the nuclear industry (*Nature* 15 October, p.505).

Congress is likely to be sympathetic. Two weeks ago the House of Representatives passed a bill that would allow utility companies to start initial low-level operations of a new nuclear plant even before all local complaints had been fully heard. A similar measure, now before the Senate, is expected to pass with little difficulty.

The economic and safety problems facing the industry are unlikely to be resolved as simply. In the past few months several local utilities have decided to abandon plans for new reactors. In several cases, such as the Pilgrim II plant which was to have been built by Boston Edison Co. on the Massachusetts coast, construction delays and the need to incorporate new safety requirements have increased initial estimates of construction costs by about a factor of ten.

Many industry supporters feel that, in view of growing consumer opposition to the rapidly escalating electricity bills to



Waiting at Diablo Canyon

finance such construction — in some cases increasing by 500 per cent within a few years — the only way for the industry to remain viable is through a massive infusion of federal funds. But this possibility is already coming under fire from both sides

Irish veterinarians in dog-house

Brussels

A report by the European Commission on the eradication of cattle diseases has levelled an accusing finger at the Irish veterinary profession. Since 1965 about £90 million has been spent by the state on eradicating bovine tuberculosis in Ireland but, says the Commission, "there has been no change in the bovine tuberculosis situation in Ireland".

"It should not be concluded that this lack of progress is due to any lack of finance (£11 million was spent in 1980) but rather to the fundamental fact that two-thirds of the reactors are being missed at annual round testing" continues the report. The Commission does not accuse anyone of cheating. It does, however, draw a comparison with the results obtained during the early 1970s by about 50 temporary veterinary inspectors employed by the Department of Agriculture to carry out tuberculin tests on a random sample of herds that were normally assigned to local veterinary surgeons for testing. The inspectors tested about 18 per cent of the national herd over several years and found approximately three times as many infected animals and herds as had the vets.

It would, of course, be to the farmers' advantage to avoid having their animals identified as being infected as this would involve high costs. Apart from the restrictions on the movement and sales of the animals and the need for proper handling

and isolation facilities, the amount of compensation paid for slaughtered beasts does not match full market value, in contrast with the situation in the United Kingdom.

Faulty testing schemes may be the answer and the European Commission, which is proposing to continue with its accelerated programme to eradicate brucellosis, tuberculosis and leukosis in the EEC, is threatening to cut off disease eradication funds to Ireland unless testing procedures are improved.

The EEC's contribution to the shared costs of accelerating the eradication of these diseases is some 130 million European Currency Units (about £65 million) over the past three years. The Commission confidently reports that by 1983 at the latest, all EEC herds will be under control as far as brucellosis and tuberculosis are concerned. By the end of the current programme, it is estimated that 1.5 million animals will have been destroyed.

Enzootic bovine leukosis will almost certainly be eradicated in Denmark in the near future and the disease is well under control in Germany. France and the United Kingdom have not availed themselves of EEC funds to eradicate leukosis. The Commission also studiously avoids contributing to the debate in the United Kingdom on the badgers and tuberculosis issue except to say that the disease has been practically eradicated there.

Jasper Becker

of the political spectrum.

The Administration has already agreed to support a federal contribution of \$123 million towards the almost \$1,000 million which it is estimated will be needed to clean up the Three Mile Island power plant. Private utilities have agreed to contribute a similar amount through the Edison Electric Institute, and the rest of the money comes from insurance cover and from the states of Pennsylvania and New Jersey.

The extra costs of new plants is not merely a result of licensing delays. As safety questions come under scrutiny, embarrassing facts are beginning to emerge. At the Diablo Canyon plant in California, for example, the wrong blueprint had been used to calculate potential stresses arising from an earthquake. And almost all of the operating nuclear plants across the country are likely to miss the deadline imposed by the Nuclear Regulatory Commission for replacing between 15 and 40 per cent of their electrical equipment which had previously been thought safe, but which was shown to be liable to failure under exposure to steam and radiation that might occur during an accident.

Such design errors have played into the hands of anti-nuclear protesters who claim that nuclear technology must remain under strict control and supervision. The industry claims that it is being strangled by these controls; but, especially at a local level the courts have tended to back up the critics.

These developments appear to foreclose all but the final NSOC option — that of increasing links with the military sector, perhaps through the recreation of the Atomic Energy Commission which shared responsibility for the military and civilian uses of nuclear fission until the early 1970s. Department of Energy officials, for example, are already claiming that their initial proposals to extract plutonium from commercial wastes through laser isotope separation (*Nature* 30 July, p.401) could partially solve the storage problem.

Here again, however, the political problems are likely to be enormous. Critics argue that allowing the military greater leverage over the civilian programme could threaten attempts to control nuclear technology through more democratic means, and that it would conflict with efforts to limit the proliferation of nuclear weapons in developing nations by trying to divorce the civilian and nuclear aspects of nuclear energy.

Nobody in Washington pretends that finding the solution will be easy. President Reagan has asked Energy Secretary James Edwards and the director of the Office of Science and Technology Policy, Dr George (Jay) Keyworth, to consult industry, the utilities and universities, and they have been given almost a year to prepare a report on "obstacles that stand in the way of increased use of nuclear energy and the steps needed to overcome them".

David Dickson

Human growth hormone Shortage persists

British supplies of human growth hormone are in danger. Over-optimism about the availability of the hormone from genetically engineered bacteria combined with a failure to appreciate that even mortuary workers are human has left the United Kingdom's National Health Service with supplies which are inadequate for the optimal treatment of the 800 British children with growth hormone deficiency.

Until biotechnology raised the prospect of an alternative, the only source of human growth hormone was the pituitary glands of cadavers. Three years ago at least 50,000 pituitary glands were collected from mortuaries in hospitals and processed in Cambridge. Another 20,000 pituitaries were collected from public mortuaries for processing in London. The combined operation, under the auspices of the

Medical Research Council, would have produced more than enough growth hormone for British needs so that up to half of the pituitaries from hospital mortuaries were stockpiled. Because of a drastic fall in the collection of pituitaries from hospitals, the stockpile is now depleted. Faced with a huge rise in cost of the hormone, the Department of Health and Social Security (DHSS) has now ordered a reduction in therapeutic dosage during 1982.

Trouble began when DHSS took over the collection and processing of pituitaries. Most of the hospital pituitaries were to be processed in the department's new Centre for Applied Microbiological Research at Porton Down. Material from public mortuaries, on the other hand, was to be handled by the Swedish company Kabi Vitrum, chosen because it has the European rights to manufacture and market human growth hormone from bacteria genetically-engineered by the Californian company Genentech. Bacterial growth hormone was to have been provided by Kabi Vitrum to DHSS at a preferential price as soon as British clinical trials, due to start in January 1981, had been successfully completed.

The first snag with these plans was that DHSS decided to use the changeover as an opportunity to consolidate into the wages of mortuary workers at hospitals, the small sum that had previously been paid to them for each pituitary collected. As many a manager might have told DHSS, consolidation can be a recipe for diminished productivity. The number collected from public mortuaries has fallen to about 13,000 a year despite the reinstatement of the special payments, but was never more than 20,000. The combined annual collection of pituitaries has therefore fallen by 60 per cent and now provides less than half the amount of hormone needed to treat British children.

The shortfall has been exacerbated by a delay in the production of bacterial human growth hormone. The first batch of Genentech's hormone to be given to humans had unacceptable side effects (fever and the lysis of blood monocytes) and full clinical trials had to be postponed. The side effects were almost certainly due to the presence of bacterial toxins in the hormone preparation, and both Genentech and Kabi Vitrum have now developed a more complex purification process. Genentech claims that its cleaner preparation has cleared toxicity tests and says that it is already six weeks into a clinical trial on children. Kabi Vitrum is slightly behind, having just completed a toxicity trial in Sweden.

Some, however, doubt whether the bacterially-derived human growth hormone will pass through its clinical trials successfully. The scepticism is based on the fact that the bacterial hormone is not quite identical with the authentic human hormone. Clever though Genentech's genetic engineers are, they have not been

Two heads for one

The Centre National de la Recherche Scientifique of France now has its complement of two heads, a director-general and a president, just two weeks after the previous incumbents resigned on a matter of principle. First — as reported last week — the mathematician Jean-Jacques Payan has been appointed director-general; and now M. Claude Frejaques, present director of the Délégation Générale à la Recherche Scientifique et Technique (DGRST) has been appointed president.

The appointments may be interim ones, as the Minister of State for Science and Technology, M. Jean-Pierre Chevènement, is said to prefer a single director for CNRS, rather than the dual headship established by the previous administration. But there was no time to change the constitution before the national colloquium, due in January, where major policy issues will be thrashed out in public, and CNRS — as the major supporter of basic research in France — has to have a clear voice by then.

Nevertheless, the appointment of Frejaques, a career civil servant rather than a scientist, has its rationale. DGRST was effectively the administration of the previous — and less powerful — science minister, Pierre Aigrain, and Chevènement has begun to set up almost a rival administration in his new ministry. DGRST may in the end be disbanded in all but name, with its parts becoming wings of the research ministry. Chevènement already has a chef du cabinet, so some role had to be found for Frejaques. CNRS seemed to suit — now leaving the minister free to shuffle DGRST as he wishes.

Robert Walgate

able to devise a way for their bacteria to produce growth hormone without an extra methionine at one end of the molecule. This amino acid, say some hormone biochemists such as Dr Philip Lowry of St Bartholomew's Hospital, is a prime target for recognition by antibodies. He therefore predicts that when used in long-term therapy, bacterially-derived human growth hormone will provoke an antibody response that will preclude its clinical use.

It is too soon to know whether Dr Lowry's predictions are right. If not, Kabi Vitrum bacterial growth hormone should be available to plug the gap in Britain's traditional supplies before dosage has to be too seriously reduced. The reduction already planned is considered not to be disastrous; one expert reckons that at worst the children so treated would end up no more than three centimetres short of their maximal height.

Because there is a world shortage of the hormone there is little hope that DHSS will find alternative supplies. And even if it could, the cost would probably be prohibitive. One estimate is that 3 years ago, the cost was £1.25 per 5-unit ampoule whereas an equivalent ampoule purchased by DHSS from Kabi Vitrum now costs £15.

Peter Newmark

UK medical research

To those that hath...

British university departments asking the Medical Research Council (MRC) for research grants will now need an assurance of financial support from their universities. MRC's chief concern is that universities, in adjusting to their own reduced budgets, may starve individual departments of funds, with the result that its research grants are "rendered ineffective".

In a letter to universities this month, the council reaffirms its belief in the dual support system, under which British university departments winning grants from research councils are supposed to be maintained as "well-found laboratories" out of the general subvention from the University Grants Committee. It acknowledges, however, that there may be temporary difficulties, as when universities decide to freeze vacant posts.

The move is MRC's attempt to force the universities' hands. Like the Science and Engineering Research Council, it is concerned that the quality of university research will be irreparably damaged if universities spread their dwindling resources too thinly. Hence it will support the objective of the University Grants Committee (UGC) that universities should concentrate their own resources on good departments considered worthy of support. Applicants for new grants from institutions not favoured by UGC may be in for a raw deal.

The MRC, nevertheless, offers some help to universities. Researchers who lose

their jobs, for example, will be eligible for small personal grants, which, while not paying their salaries, will help them complete projects already started. Universities are also invited to nominate exceptionally promising researchers who may be eligible for help. MRC is willing to expand its senior fellowship scheme which provides support for up to ten years for promising young researchers unable to find tenured posts. Demand for the scheme, which has been running for four years, is already heavier this year than in the past. MRC is also willing to increase the number of awards it makes under existing schemes which free academics from teaching and administrative tasks to allow them to spend a few years on full-time research.

The release of the MRC letter to the universities last week coincided with publication of its annual report for the year ending March 1981 (HMSO £4.00). According to the report, the council has resolved its long-standing dispute over the terms of employment of researchers on short-term contracts. The council has agreed that 70–80 per cent of its posts will carry tenure, compared with 67 per cent previously. Short-term appointments will now be almost exclusively for three years and open competition will be invited for tenured positions. The new scheme will not cost the council more, according to Dr James Gowans, Secretary of MRC, chiefly because it does away with appointments of intermediate term.

During 1980–81, the council spent nearly £93 million, about £15 million of which was transferred to its budget from the Department of Health after the collapse of the Rothschild customer-contractor principle. Under the principle, first introduced in 1974, money was transferred from the council's annual budget to government departments for spending on research commissioned through the council. The new policy, however, has made little difference to the council's work, according to Dr Gowans, because the health department chose to commission long-term research which the council will continue to support. In the deal struck with the health department, the council has agreed to increase support for health service research to £2 million by 1985–86.

The year to March 1981 was not easy — the council had to supplement its government grant with £500,000 of its own money and was not able to provide all the support for top quality research for which it was asked. But Dr. Gowans's chief concern is for the future. In particular, he fears that the government may renege on its earlier promise to maintain the real value of the science vote to be announced in December. The implications of a real budget cut for MRC, which in any one year has more than 90 per cent of its budget tied up in on-going commitments, could be far-reaching, involving a reduction in the amount of research it supports in universities.

Judy Redfearn

European Science Foundation

Signs of solidity

Strasbourg

The European Science Foundation (ESF) — Europe's fledgling international academy — appears to have come of age. Last week the foundation, representing 47 research councils in 18 countries, made its first direct approach to governments with a letter to research ministers requesting them to take up the idea of a "European Synchrotron Radiation Source", a £30 million third-generation source of X-rays for Europe.

The new source is considered necessary by most European X-ray users if Europe is to keep abreast of American competence in the field. But the foundation's request is significant not only for its content but as an example of the foundation's new confidence in the practical role it can play in Europe.

The foundation has previously been cautious of its role *vis à vis* its member research councils, but there seems to be such agreement among the various councils over the synchrotron source, such a need to cooperate financially in the present recession and such growing confidence in the foundation's offices that the members are willing to let the corporate ESF approach go ahead.

Professor Hubert Curien, the French president of the foundation, makes it clear in his letter to the ministers that ESF — as a non-governmental organization — cannot handle political questions such as the site for the source or the national contributions to its cost, but requests governments to set up a committee of representatives to do just that. The governmental committee would then work at arm's length from the foundation, "seeded", as it were, by the foundation's earlier enthusiasm, hard work and the now-detailed specification of the X-ray machine.

How governments will respond is yet to be seen, but already several countries and organizations have made unofficial offers of sites, the most detailed of which has come from Italy (for Trieste). The ESF committee has also made a careful study of the possible use of a tunnel at the European Centre for Nuclear Physics (CERN) near Geneva, which now holds the intersecting storage rings, a ten-year-old device likely to be closed within a few years to save money for the large electron-positron collider (LEP). If the new X-ray source were built at CERN it would, however, come outside the CERN budget.

According to the foundation's optimum timetable, the intergovernmental committee would meet early in 1982; governments would take a decision in principle in early 1983; and the source would be in operation by 1988–89.

The foundation also agreed last week — at its annual assembly in Strasbourg — on a serious study of what may be its next big project — a "geotraverse" of Europe. This

would entail a detailed geological survey down to the mantle, using every available technique, of a band 2 to 20 kilometres wide stretching from the North Cape in Norway to North Africa, across the many major ancient geological boundaries of Europe.

The exercise would be a continental parallel to the International Deep Sea Drilling project and would cost around £3 million over seven years, 2–3 years of which would be used to gather data. The Swiss national research council will pay for a pilot study.

On a smaller scale, the foundation has decided to set up a new fellowship scheme — this for toxicologists — similar to its existing European Training Programme in Brain and Behaviour Research. Each year £70,000 will be made available by council in nine countries (Denmark, Finland, Ireland, Italy, The Netherlands, Norway, Sweden and the United Kingdom) for short-term and long-term fellowships in toxicology. The object is to stimulate research on the toxicology of environmental chemicals — and to help increase the number of experts in Europe who could advise governments and industry. The first fellowships will be advertised next spring to be available in autumn 1982.

Robert Walgate

EEC Research Council Broader future

Brussels

EEC's ten research ministers have finally approved the first stage of a biomolecular engineering programme and the 4-year programme on microelectronics (worth \$40 million). This agreement, reached at a meeting on 9 November, reflects the future attitude of the Community to research and development.

The European Commissioner for industry and now research, Etienne Davignon, sees research and development as being one of the prime vehicles by which Europe's flagging industrial competitiveness compared with the United States and Japan can be revived. That his ideas are being taken seriously by member states is demonstrated by the council's decision to go ahead with biomolecular engineering and microelectronics programmes.

Only the first stage of the 4-year programme (indirect action) on molecular engineering has been agreed. The original six comprehensive projects proposed by the commission still stand, but the whole programme will now be focused on agriculture and on safety and environmental questions. So for two years and with about \$8 million to spend on 50 per cent support, the commission will fund research on, say, the synthesis of vaccines and pesticides of importance to European agriculture; on the biotransformation of agricultural surpluses and wastes; and on plant molecular genetics and gene transfer. The safety work — accounting for 20 per

cent of the grant — will cover the detection of contaminants in industrial microbial strains and the extension of risk assessment procedures.

After two years, the programme will be re-assessed and if successful continued — probably with a further injection of cash. The commission hopes to call for tenders around the end of this year, and the programme will start in earnest on 1 April 1982.

The commission's 4-year action programme in microelectronic technology is the second arm of the strategy to stimulate European research into telematics and informatics. A programme has been under way since September 1979 on data processing and a third programme on telecommunications is expected to be proposed before the end of 1981. The agreed budget is for \$40 million, \$12 million less than was originally asked for.

This programme is also important because member countries have agreed to coordinate their activities and keep each other informed of new developments to ensure that a microchip production industry is soon established in Europe.

Figures from a report being prepared on the competitiveness of European industry illustrate the struggle facing Europe. Jobs created in Europe between 1970 and 1980 numbered 2 million compared with 19 million in the United States and 5 million in Japan. Japan spends globally half as much money on research as EEC, but Japanese researchers register four times as many patents.

In the field of microprocessors, EEC is calculated to have spent \$470 million developing chips compared with Japanese expenditure of \$240 million. But Japan and the United States each supply 40 per cent of the world microprocessor market, while European production accounts for less than 10 per cent.

The commission's desire to coordinate research efforts carried out at national levels would involve holding regular twice-yearly meetings to plan and exchange information and analyse national spending. By discussing programmes at the early stages, overlapping and duplication could be avoided and lead to an efficient dissemination of research results both among the member states and between universities and industry. Using Euronet as an industrial data base and the planned INSIS integrated numerical network, the gap between research and industrial application would be narrowed.

For the Community's joint research centres, Davignon foresees the scope of the research being widened — a move that might involve the opening of the centres to agricultural research for the African, Caribbean and Pacific countries linked to EEC by the Lomé convention. The concept of promoting "centres of excellence" is also being discussed.

Agriculture research will also be boosted. Only 1.1 per cent of EEC's

research budget is devoted to this field despite the vital role the Common Agricultural Policy plays in EEC affairs.

Although the commission seems to be backing the argument that increased research and development is a means of solving current economic problems, a belief supported by European industrialists, ministers were non-committal on Davignon's request to double between now and 1986 the amount of money from the Community budget actually devoted to research and development.

Jasper Buker

Netherlands universities

Misery ahead

Ending several weeks of uncertainty the Netherlands government announced on Monday the latest forward plan for the universities. Briefly, the Ministry of Education and Science is looking for a 2 per cent cut in university salary budgets in the years 1984 and 1985, together with a 3 per cent cut in other expenditure. Although the percentage reductions of the university budget (expected to save a total of 75 million Dutch guilders (£16.5 million) a year) are not at first sight large, coming as they do after several years in each of which university budgets have been reduced by 3 per cent, the consequences could be serious.

In the two years ahead, the ministry has also decreed that there should be a freeze on academic vacancies. During that period, the ministry also hopes that there will be a rationalization of the structure of university departments, with resources concentrated in the stronger departments. The Academic Council, which advises the ministry, has already begun to put individual departments in order of merit. It is possible that if the universities concerned do not take the initiative in reorganizing themselves, the minister will provide an incentive by adjusting the grants they are offered in the years ahead, either up or down.

One curious feature of this week's proposals is that the government expects the universities collectively to pay their bills less promptly. The result may be that the drain on the government's cash resources is reduced by up to 40 million guilders in 1982. The reactions of the universities' creditors are not yet known.

On the face of things, there will be no immediate need of redundancies among academic staffs, although the ministry has set up a central register of vacancies. Even during the two years ahead, universities will be free to apply for a dispensation to fill vacant posts considered essential to their academic or research programmes. There is, however, a possibility that some universities will prefer to reduce their staffs than to stomach for a further two years the acute shortage of disposable income from which they have been suffering.

The next step will be for the parliament

in the Hague to approve the two university budgets — for teaching and for research — now proposed. Further support for research projects is provided by the Netherlands research council (ZWO) whose financial importance in university affairs has been increasing in recent years.

However, ZWO will have to do without a science minister — a post established only eight years ago. The previous minister, Anthonius van Trier, was without portfolio, but he badgered his ministerial colleagues into giving him control of substantial parts of their budgets. The new government has removed this irritation, the support of science reverting to the new minister of education, Josephus van Kemenade. And as Kemenade's major political interest is in establishing comprehensive education for Dutch schoolchildren, scientists in the Netherlands are worried.

Agricultural research

Changes mooted

Washington

"Reach out and touch somebody", the slogan being used in the United States to promote the use of long-distance telephone calls, has also become a newly-prominent policy theme for the thirteen agricultural research institutions which constitute the Consultative Group for International Agricultural Research (CGIAR).

The group is an informal network organized under the auspices of the World Bank through which developed countries, multilateral agencies and private foundations channel their support for research at the institutions into developing countries' agricultural needs.

At their annual meeting in Washington last week, both donors and research administrators endorsed current efforts by the institutions to increase linkages with outside research workers in two directions. One is to strengthen collaborative research projects with universities and other institutions in developed countries, in order to make the best use of present scientific knowledge. The second direction is to improve links with national agricultural research programmes in the developing countries.

It was the tenth annual meeting of the group, which has grown from four to thirteen members since it was founded in 1971. Since then, the total amount of funds channelled through the CGIAR system has risen from \$20 million to \$135 million.

In the past few years, however, as the rate of inflation has crept upwards, the growth of the institutes in real terms has begun to slacken off. Last year, for the first time, several institutes had to trim their programmes when it was realized that with various industrialized countries — and private institutions — cutting back on their aid programmes, voluntary contributions would not meet the targets.

Sowing more seeds

Mexico City

One of the biggest and best known of the research centres funded through the Consultative Group on International Agricultural Research (CGIAR) is the International Maize and Wheat Improvement Center (CIMMYT, after its Spanish initials), whose headquarters nestle in foothills on the edge of a high, fertile plain thirty miles north of Mexico City.

Based on a programme initially set up by the Mexican government and the Rockefeller Foundation in the early 1940s, CIMMYT was formally established as an international centre in 1966, and was one of the four founding institutes when CGIAR was created in 1971. Its current budget makes the largest financially of the 13 centres which now constitute the group.

In terms of its initial mission — the use of scientific breeding techniques to increase the yield of wheat and maize crops — CIMMYT has been spectacularly successful. It is known internationally as the home of the Green Revolution, due to the high-yielding varieties of dwarf wheat for which its most famous scientist, Dr Norman Borlaug, was awarded the Nobel prize. Among its more recent success stories is Bangladesh, which has increased its wheat yield from 114,000 tons in 1975 to 1.2 million tons in the current year.

But times are changing at CIMMYT. Although the mainstream research continues along conventional lines, CIMMYT is increasing its efforts in "farm systems research", addressing questions such as crop management and agricultural economics. Greater emphasis is being placed on the development and dissemination of improved research procedures to national research programmes, as well as on the support of indigenous training programmes.

This year the situation looks as if it will be even tighter. Pledges for donations made at last week's meetings totalled \$155 million, an increase of about 15 per cent over the current year. However, according to Mr Warren C. Baum, the present high inflation rates plus fluctuations in the exchange rates covering the currencies in which donations are made mean that real growth will be small.

The combination of financial stringency and structural changes in the international research environment stimulating various shifts in strategy. One has been to increase the emphasis on basic research either at the institutions themselves or through links with the international scientific community.

Complementary to this will be the efforts

One of the more controversial issues the centre faces is the growing demand on both sides of the Atlantic for greater patent protection for plant breeders. Dr Borlaug, acting director of CIMMYT's wheat programme, has criticized the new legislative initiatives, arguing that they could make developing countries more vulnerable to exploitation by unscrupulous outside companies. But others at CIMMYT seem prepared to accept, if reluctantly that tighter patent rights could accelerate the dissemination of new agricultural technologies.

The changing economic dynamics of food production are producing their own tensions within CIMMYT. Until now the centre has been distributing its germplasm with no charge and virtually on request. But after recent incidents, the rules are being tightened up.

Some recent scientific developments are also being watched warily. For example, there is scepticism about the size of the potential contribution of recombinant DNA technology to the direct improvement of crop yields, and CIMMYT has no molecular biologists on its staff. "We think that Wall Street is being widely optimistic", says CIMMYT's director general, Dr Robert D. Havener, referring to the heavy investor demand for shares in the new genetic companies, and quoting Dr Borlaug's view that "it will be 50 years before there is any significant impact on complex plants coming out of genetic engineering".

At the same time, however, CIMMYT is strengthening its links with research scientists in developed countries so that they can exploit any major breakthrough. Meanwhile Dr Havener's principal concern is to ensure that political and economic pressures do not upset the arrangement under which centres such as CIMMYT operate with minimum outside interference and the maximum amount of flexibility.

David Dickson

to assist national research programmes. In the past, tensions have arisen when donors faced difficult choices over whether to allocate funds to a particular country's research effort or to the international institutions. At a meeting held earlier this year in Nairobi, Kenya, for example, representatives from several African countries expressed strongly their view that CGIAR as a group should be doing more to assist indigenous efforts in their region, a message which is now being acted upon.

Keen to maintain the minimum of bureaucracy, last week's meeting reacted equivocally to a suggestion from the review committee that a new committee be established to coordinate budget request and allocations, and the issue has been deferred.

David Dickson

CORRESPONDENCE

Animal committees

SIR — Your article "Protection for laboratory animals?" (*Nature* 17 September, p.173) presents a rational and realistic appraisal of the present dilemma concerning the ethical use of animals in experimentation being faced by the scientific community, the animal welfare community and the legislator.

It was of significant interest to the Canadian Council on Animal Care (CCAC) that the article concluded with the suggestion of building into a new system of regulation the establishment of "a committee at every important centre at which animals are used that would be empowered to sanction (or not to sanction) proposed uses of laboratory animals". The CCAC, established as a committee of the Association of Universities and Colleges of Canada in 1968 "to work for the improvement in the care and use of animals on a Canada-wide basis", has had, as its cornerstone, the development of local committees on animal care, at every institution in the country using animals in research. It was, and is, the intention that this committee should act as the "conscience" of the institution in ensuring ethical animal use is practised.

We recognize that we are dealing with human beings whose abilities and motivation vary. Therefore, some committees are more effective than others. However, it is our objective to continue to monitor the activities of these committees, in order to ensure that the weaker ones are made strong, and the stronger ones continue to be strengthened.

It is our opinion, and one based on experience, that a local committee comprising conscientious and strongly-motivated members can do more than legislation to ensure the ethical treatment of animals in research.

H.C. ROWSELL

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Ottawa, Ontario, Canada

On chemical war

SIR — As noted by David Dickson (*Nature* 1 October, p.327) and Philip Campbell (*Nature* 22 October, p.598) the last few months have seen a potentially dangerous escalation in chemical warfare. This escalation was symbolized by the Reagan Administration's decision in February to build a new nerve gas munitions plant in Arkansas, approved by the Senate at the end of May. US stockpiles of nerve gas already stand at well over 10,000 tons and while Warsaw Pact stockpiles certainly exist, their quantity is not known. France also has the weapons. The United States is known to have been pressing for a British contribution to its build-up, whether by stock-piling US weapons or other means, even though our government continues to endorse the policy which led to the disposal of the British stockpile in 1957 and the decision not to develop a replacement. Chemical warfare is prohibited by the Geneva protocol and customary international law.

A new chemical arms race in Europe would be an additional hazard over and above that already presented by nuclear weapons. Furthermore, it would impose new military direction and priorities on research and development in the life sciences and

biotechnology. Currently, the Ministry of Defence is in contract with British universities to the tune of £5 million per annum. With bleak prospects in front of them, and the reduced level of funding by the research councils, university departments may be tempted to seek defence contracts and acquiesce to all that these entail.

We therefore call on scientific and technological colleagues not to participate in research associated with the development and production of chemical weapons; to urge the British government to forgo the production and stockpiling of chemical weapons in the United Kingdom; and to press the government to:

- (1) Withdraw its reservation of the right to retaliate in kind made by Britain when ratifying the 1925 Geneva Protocol.
- (2) Resubmit the draft Chemical Weapons Convention tabled by Britain in 1976, revised to incorporate new proposals on verification, consultation, scope and confidence-building measures.
- (3) Promote specific negotiations on the withdrawal of chemical weapons from both sides of Europe.

A significant start in this direction has already been made, namely the support of 2,000 colleagues, including five Nobel laureates, in science, technology and medical faculties throughout Britain. Further support would be welcome.

SEAN MURPHY

ALASTAIR HAY

JULIAN PERRY ROBINSON

Open University, University of
Leeds and University of Sussex, UK

Cancer monoclonals

SIR — The use of monoclonal antibodies in the treatment of cancer is at present being assessed and there is a section of the biomedical community, both scientific and administrative, that believes empirical clinical trials of monoclonal antibodies and toxin-antibody conjugates should be conducted immediately. I wish to present the arguments for a more orderly, scientific and cautious approach.

Three major points can be made in support of the latter case. The first is scientific: it is important to gather as much information as possible before clinical trials with monoclonal antibodies about the tissue distribution of the target antigen, chemical structure and so on. This is for two reasons. (1) The factors involved in either the success or failure of serotherapy cannot be assessed without this basic information. (2) The potential risks or benefits to the patient cannot be accurately measured — this is illustrated by two recent publications. The first reported the (unsuccessful) use of a monoclonal antibody against CALLA antigen in the serotherapy of acute lymphoblastic leukaemia¹; the second that the CALLA antigen was not confined to lymphoid cells but was present on several other tissues, particularly in the kidney.²

The second argument is ethical. The minimum right of a terminally ill cancer patient subjected to experimental therapy is that every effort has been made to assess the potential deleterious effects of the proposed therapy. Moreover, it can be argued that the

patient should also expect the potential benefits of the treatment to outweigh the potential risks.

The final argument against empirical therapeutic trials of monoclonal antibodies before extensive preclinical testing is that of the relationship between the biomedical community and the general public. In the past, cancer patients and their families have had their expectations of more effective treatments for cancer raised unrealistically by the early optimistic reports of clinical trials with transfer factor, BCG and, more recently, interferon. The biomedical community would be well-advised to avoid a similar situation with monoclonal antibodies.

IAN S. TROWBRIDGE

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1. Ritz, J. et al. *Blood* 58, 141-152 (1981).
2. Metzgar, R.S., Borowitz, M.J., Jones, N.H. & Dowell, B.L. *J. exp. Med.* 154 1249-1254 (1981).

Room for Anon

SIR — I see that you have again used the word "culprit" in connection with users of the pseudonym Isadore Nabi (*Nature* 29 October, p.696).

Although I refrained before, I would now like to express my conviction that the use of a pen-name, in publishing a work of literature or science, is not of itself a culpable act. Indeed, the dangers would seem to be least in theoretical science, where such contributions can normally be judged on their intrinsic qualities.

As your readers may already be familiar with literary examples, such as that of the Brontës, I will recall a few examples from mathematics.

(1) In statistics, "Student's test" is so known because it was originally published over the pen-name "Student". The author's true name is known to a few historians. Who says he did anything wrong?

(2) In *Annals of Mathematics* (69, 247-251; 1959) we find a letter purporting to come from a mathematician long since dead, saying how glad he is to see his results discovered independently by the mathematicians of the present day. The letter convinces me of the present day author's standing both in mathematics and in the history of mathematics; and he chose an amusing way to make his point. Who says he did anything wrong?

(3) The pen-name "N. Bourbaki" is used by a French mathematical syndicate, who have published some very useful books and gained a considerable reputation.

(4) There is some tradition that serious mathematicians, if they write an occasional piece which is recreational or humorous, may shelter behind pen-names such as "H. Petard".

Your mathematical readers can find out which journals I help to edit. I would like to assure them that I shall treat pseudonymous contributions just like any other contributions — on their merits.

J.F. ADAMS

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Fast breeder reactors: can we learn from experience?

Otto Keck*

INITIALLY, developing the fast breeder reactor (FBR) carried the promise of a virtually inexhaustible source of electricity at competitive cost. Now, three decades and substantial amounts of public expenditure later, we are not much closer to the fulfillment of that promise. Economic evaluations have consistently proved too optimistic. The commercial use of FBRs has been pushed back year after year. This experience gives little comfort to policymakers who have to decide about the next steps in FBR demonstration and commercialization.

Can we identify the sources of over-optimism and improve future policymaking? A close examination of the West German FBR programme, drawing on interviews with about 40 participants and the documents of government advisory bodies, shows a way to bring more realism into policy decisions¹. The findings suggest that governments can redress the optimistic bias by requiring reactor manufacturers and utilities to make an increased contribution to the cost of the programme from their own funds. In particular, construction of a commercial-size demonstration plant should be made contingent on the utilities' willingness to finance it from their own funds. Does this proposal take due account of the potential long-term benefits to be expected from the FBR? An economic analysis of FBRs suggests that the answer is yes.

Precarious beginning

In West Germany, the development of the FBR began late compared with other countries. The principle of nuclear breeding was discovered in the 1940s, during the United States' effort to develop the atomic bomb. In 1951, a small American FBR produced the first nuclear electricity. By that time the Soviet Union and Great Britain had started on their own programmes.

The initiative for the West German programme came from the Karlsruhe nuclear research centre, a laboratory founded in 1956 with the objective of constructing and operating the research reactor FR2. Although industry contributed about half the construction costs of the reactor, the laboratory was basically

a government organization. When the design of the FR2 reactor was nearing completion, the Karlsruhe scientists looked for new tasks. New work was particularly urgent for the nearly 100 scientists and engineers in the technical division, who faced lay-off after completion of the FR2 reactor. The scientists' interest focused on the FBR and a project group was established in April 1960.

The experience of the West German fast breeder reactor programme suggests ways of bringing more realism into governmental decisions on the development of new reactor types. In particular, reactor manufacturers and utilities should finance commercial-size demonstration plants from their own funds.

A preliminary programme for three years costing DM 25 million (£2.1 million at 1960 exchange rates) was endorsed towards the end of 1960 by an advisory committee of the then Ministry for Atomic Energy. When presenting its plans, the Karlsruhe team referred to American and British forecasts that FBRs would assume a significant commercial role soon after 1970. But the advisory committee by implication rejected these forecasts when it stated explicitly that work on FBRs might be terminated after the preliminary three year programme. This cool reception came mainly from committee members with industrial backgrounds. Ministry officials were very much in favour of the project and the DM 25 million was only a fraction of what the ministry was prepared to spend for a rapid expansion of the Karlsruhe laboratory.

Boost from Euratom

The threat of possible termination did not worry the fledgling Karlsruhe project for long. Coincident with the advisory committee's cool appraisal, the European Atomic Energy Community (Euratom) began to show interest in FBRs. The community had been founded in 1958 by Belgium, France, West Germany, Italy, Luxembourg and the Netherlands. It had little success in associating itself with national programmes for first-generation nuclear power plants (light-water reactors [LWRs] and gas-graphite reactors). To secure a useful function, Euratom committed its considerable funds to exotic technological niches, such as the organic-cooled reactor, and to advanced designs, such as FBRs.

In 1963 the West German government signed an association contract with Euratom that gave the German FBR

programme the benefits of Euratom money without taking away domestic control. Similar contracts were made in support of the French programme begun in the late 1950s, and of the Italian programme, for which the Euratom contract served as a primer. For the Karlsruhe team, the association implied authorization of an expenditure of DM 185 million (£16.6 million), extending the

project up to 1967. Euratom was to contribute 40 per cent of these funds. This commitment made FBRs the main objective of the Karlsruhe laboratory and gave this reactor type the first priority in the West German reactor programme. In the period 1956–67 — before the first commercial orders for LWRs were placed — FBRs received more government support than any other reactor type.

This shift in priority was made without a fresh consideration of the economic need. For the Karlsruhe project, the Euratom association was a matter of survival. The Ministry for Atomic Energy was motivated mainly by the political aim to secure a fair return from West Germany's contributions to Euratom. In favour of this, it ignored the earlier cool appraisal of the FBR by its advisory committee.

Prototype design

In September 1964, the Karlsruhe team was shocked by the announcement by General Electric (US) that the company would offer a commercial FBR as early as 1974. The scientists felt that the West German programme must be pushed ahead as fast as possible to meet this competitive threat. They therefore proposed that work should start immediately on the detailed design of a 200–300 megawatt prototype plant two years earlier than previously planned.

At that time, the laboratory was working on two different versions of the FBR — one using sodium as coolant, the other steam. As a choice between the two seemed difficult, the scientists proposed designing and building one prototype plant of each type. The Ministry for Scientific Research, successor to the Ministry for Atomic Energy, awarded design contracts in November 1966 to two industrial consortia. It allocated DM 57.5 million

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(£5.1 million) for industrial work on the sodium-cooled prototype and DM 38.7 million (£3.5 million) for the steam-cooled prototype.

Apart from the threat of international competition, Karlsruhe's justification for accelerating the programme rested on estimates of electricity costs suggesting that, in the 1970s, FBRs would produce electricity for 15 per cent less than LWRs. The advisory committees did not discuss either argument explicitly. Implicitly the arguments were rejected when reactor manufacturers and utilities declined to make a contribution to the programme from their own funds, arguing that the economic use of FBRs was not foreseeable. Nevertheless, the ministry adopted the Karlsruhe estimates.

Termination of steam breeder

At the end of 1967, the industrial team working on the steam-cooled prototype discovered technical problems that prevented conversion of a small boiling-water reactor into a facility for testing FBR fuel elements. When plans for a 50 megawatt steam-cooled FBR in the United States were dropped in 1968, the ministry acknowledged that the ensuing lack of an information exchange with foreign programmes would increase the risks and costs of its own programme. It therefore decided in 1969 to abandon the steam-cooled FBR.

At first, the firms involved opposed termination. However, the ministry got their consent by asking them either to finance part of the further development costs or to agree to the termination. The

firms preferred to spend their money on other projects.

SNR-300

The economic assessment of FBRs reached a turning point in West Germany when contracts for the 300 megawatt sodium-cooled prototype plant SNR-300 were negotiated. Quotes for construction cost were four times more than earlier estimates (excluding inflation). Fuel fabrication costs were quoted as about ten times the previous estimates for commercial FBRs. This ruled out any possibility of FBRs undercutting the electricity costs of LWRs as long as uranium from low-cost reserves was available.

A new economic assessment by an advisory committee concluded that global low-cost uranium reserves might be depleted around the year 2000, so that FBRs would not be needed before the 1990s. Probably two demonstration plants would have to be built after SNR-300 to bridge the gap to commercialization.

Postponement of the SNR-300 might have saved money by shortening this gap. However, changes in the schedule were difficult because the programme had acquired institutional momentum. A large proportion of the Karlsruhe laboratory as well of supplier firms was engaged on the FBR. If the teams were to be kept together, construction of the SNR-300 had to start within a relatively short time.

Construction began in 1973 with only two years delay against an earlier schedule. Belgium and the Netherlands participated in the project, each contributing a 15-per cent share. In 1973, the SNR-300 was

reckoned to cost DM 1,335 million (£205 million), excluding the plutonium fuel. Operation was planned to begin in 1978. Since then, costs have more than doubled (in constant prices). The latest estimate as of February 1981 gives a figure of DM 5,000 million (£1,050 million). Completion is now expected in 1986.

Nearly all the funding for the project has been and is being provided by the three governments. The utilities contribute about 8 per cent of the construction cost from their own funds. West German manufacturing firms finance 8 per cent of the related research and development they perform. The total cost of industrial research and development for the SNR-300, which is not included in the above figures, is estimated at DM 350 million (£75 million). Recently, the German utility participating in the project promised to contribute an additional DM 31 million both in 1981 and 1982, and the German reactor manufacturer pledged an amount of DM 10 million in each of these years. Even so, the firms' contributions to the overall project cost will still be less than 10 per cent. The governments also pay for all research and development performed in government laboratories. For West Germany, this implies an additional estimated expenditure of DM 950 million (£200 million) over the period 1973–82.

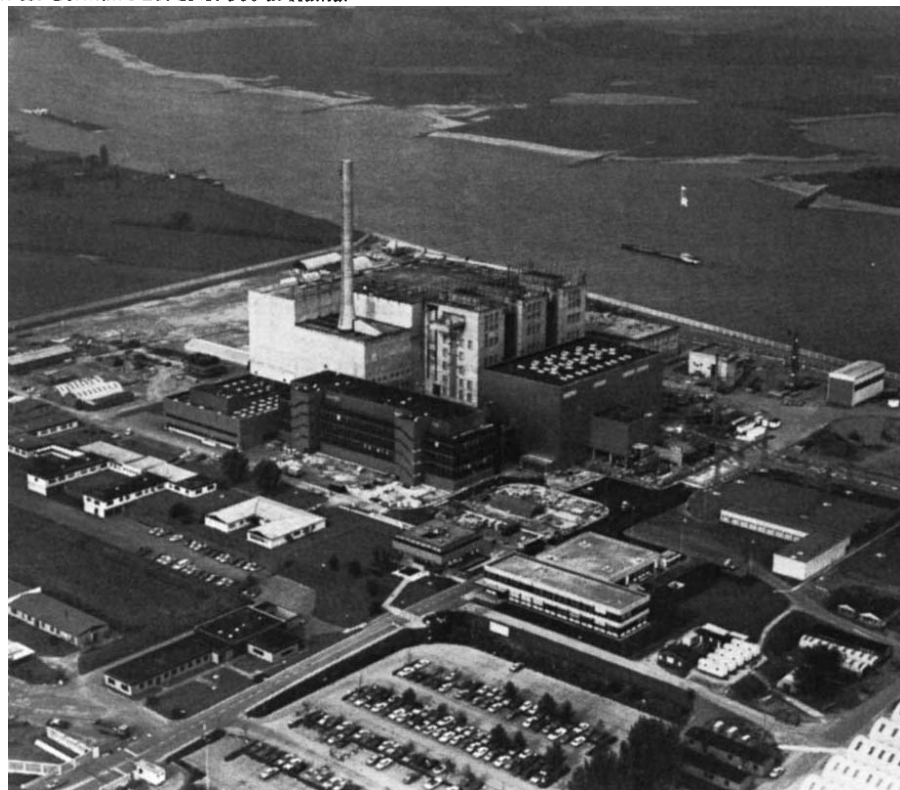
Lessons

The West German experience suggests that government laboratories and agencies are ill equipped for economic assessments. As they are remote from the commercial world, they may misread the information provided by industry or the technical literature. Organizational needs or political considerations often conflict with a realistic economic evaluation — and when they do, government organizations are tempted to opt for the former.

Reactor vendors and utilities have consistently made more realistic economic assessments than the governmental participants. Yet only at the outset did representatives of industry in the advisory committees volunteer their scepticism, and even that was prompted by the general dispute on the division of responsibilities between government laboratories and industry. Later, firms revealed their scepticism only when asked to contribute some of their own funds. Then they did so implicitly rather than explicitly, revealing it only to the extent that was necessary to defend the limits on their willingness to put up their own money.

As long as only government money is at stake, industrial participants in joint projects seem to have little incentive for a sober review and frank exposure of commercial and technical uncertainties. Criticizing economic assessments made by other organizations is unpleasant. Firms do not wish to exclude themselves from a government-financed programme by questioning the government's economic

West German FBR SNR-300 at Kalkar



justification. Indeed, they want to be part of the programme to know what is going on and to get some of the business.

These conclusions support the proposal² that the construction of commercial-size demonstration plants should be contingent on the willingness of utilities to finance the costs from their own funds. A near-commercial framework should be applied that would require reactor manufacturers to back cost estimates by guarantees with respect to performance and cost, hence making them participate in the technical and commercial risks. Although there is a case for a greater role of government in the earlier phases of a development programme, at the demonstration stage government support should be restricted to the sharing of operating risks and to the financing of background work performed by government laboratories.

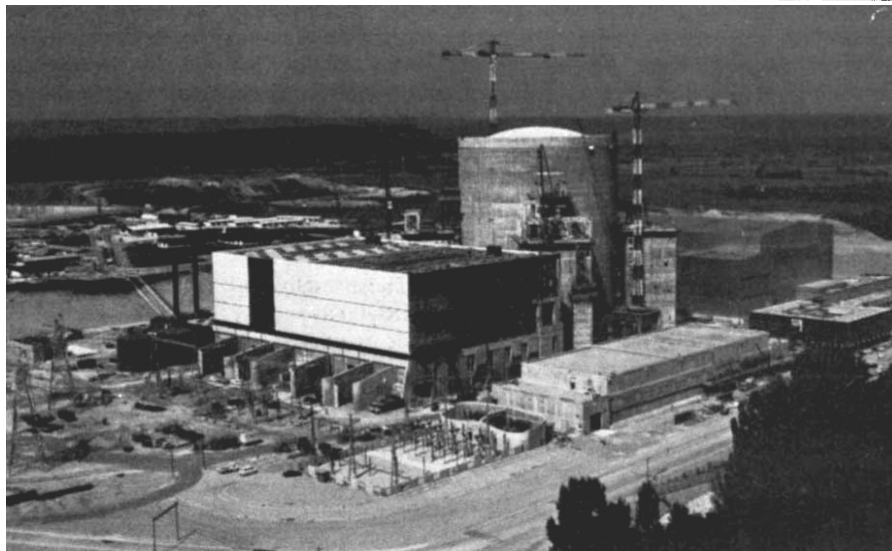
Whether demonstration plants are financed through taxes or utility bills, in the end the citizen will have to defray the cost. But the government is likely to get more realistic advice if it asks utilities whether they are prepared to charge the full cost to their customers' electricity bills.

Long-term benefits

Against this proposal it may be argued that the FBR may become an indispensable source of energy at some time in the future that is further away than the short-term calculations of utilities can accommodate. A demonstration plant has to be constructed soon, the argument goes, if we are to have the capability of constructing a larger number of FBRs ten to twenty years from now, when there may be a need to do so because of the depletion of uranium resources. A study by the Organisation for Economic Cooperation and Development (OECD) and the International Atomic Energy Agency (IAEA) estimates that at a cost up to \$130 per kilogramme there are about 5 million tonnes of uranium available in the non-communist world (reasonably assured and estimated additional resources). According to the growth projections for nuclear power by the International Nuclear Fuel Cycle Evaluation (INFCE), this may not satisfy demand beyond the year 2010.

But such calculations do not justify paying the penalties involved in constructing a FBR demonstration plant that is more expensive than what a utility would be willing to pay. Uranium prospecting and exploration have a very short history, and vast areas remain to be investigated. On the basis of indirect indications and of geological extrapolations, another study by OECD and IAEA has suggested that an additional 7–15 million tonnes of uranium deposits may be discovered with existing exploration techniques. These may carry nuclear power to the middle of the next century, if a modest growth of nuclear power is assumed.

Past projections of uranium supply and demand have proved an unreliable basis for



French FBR "Super-Phénix" at Creys Malville

policy decisions. Forecasts of future nuclear capacities have steadily been downrated, while estimates of uranium resources have continuously been increased. The lower of two growth projections for nuclear power published by INFCE appears unrealistic only one year later. We do not know how long this trend will continue. The nuclear industry in most countries is presently more concerned about its survival over the short term than about the long-term supply of uranium.

Given the large uncertainties in the long-term demand for uranium and the usual conservatism in resource estimates, there is no assurance that depletion of low-cost uranium resources will necessitate the use of FBRs within the next fifty years. Thus a prudent policy will take into account that depletion of uranium resources will not occur suddenly. As low-cost resources become scarce, the price of uranium may eventually rise to a level that may make the use of FBRs an economic proposition. Uranium resources are likely to be available in sufficient quantity for a transitional period until FBRs replace current types of nuclear reactors. Because of the long lead times of nuclear plant construction, the demand for uranium two to three decades ahead can be fairly easily assessed. The penalties for missing the right time for a transition to FBRs by a decade or two is rather small, as the cost of natural uranium accounts for less than 10 per cent of the total electricity costs in LWRs.

Once reprocessing is available on a larger scale, advanced core designs may become feasible for the LWR that greatly enhance its fuel efficiency. A study by Kraftwerk Union and the Karlsruhe laboratory, for example, proposed a core that uses five times less fuel than present designs. If such designs have lower electricity costs than FBRs, they may delay the need for FBR commercialization by decades.

Premature construction of an uneconomical FBR demonstration plant may also be advocated as an insurance against

short-term interruptions of uranium supply. However, one FBR demonstration plant alone would make a negligible contribution, and construction of a series of uneconomical FBRs may provide the desired insurance at a much higher price than other options. A fully developed LWR fuel cycle is little affected by interruptions of two or three years. Long-term supply-contracts can greatly increase the reliability of the international uranium trade. If additional insurance is desired, stockpiling and preparations for emergency mining of low-grade domestic ores or for extracting uranium from seawater may be less expensive options than a series of uneconomical FBRs. Once the technical feasibility of advanced LWR cores is demonstrated and reprocessing is available on a larger scale, this technology will be a strategy, as virtually no insurance premium cost will have to be paid against the threat of a uranium shortage.

Economic uncertainties

The perspective that the SNR-300 throws on FBR electricity costs suggests that economic uncertainties are several and large. According to the latest estimate in February 1981, the SNR-300 costs about seven times more per kilowatt of net electric output than a commercial LWR, which was offered at that time at a price of about DM 2,550 (£540) per kilowatt (including owner's cost, but excluding the first core and the interest, inflation and taxes during construction). Scaling-up to sizes around 1,300 megawatt would reduce the cost per kilowatt of the breeder by 40–60 per cent. But this would still imply FBR capital costs 2.7 to 4.3 times greater than LWR costs.

The firms constructing the SNR-300, nevertheless, argue that this plant may not be representative of future large FBRs. In particular they say that:

- The cost includes a good deal of research and development that will not have to be repeated for future plants.

- The SNR-300 design is sub-optimal with regard to cost, since design changes imposed by the licensing authorities had to be incorporated into a largely fixed plant concept.

- The supplier's engineering capacity was underutilized as it could not be deployed for other projects during the construction delays caused by the licensing procedure.

These arguments suggest that by extrapolating from the SNR-300 data we may overstate the construction cost of future large FBRs. But we do not know by how much. If engineering services are excluded from the SNR-300 cost estimate, my calculation still suggests FBR capital costs at least double those of the LWR. On the other hand, the costs of SNR-300 are bound to increase further before the plant is completed. As for the next few large FBRs, it is highly probable that most of the factors that have increased the cost of the SNR-300 will still apply, although perhaps to a lesser extent. These factors will become insignificant only if a series of nearly identical plants is constructed at the rate of about one a year.

The hopes of the FBR community now rest on the French 1,200 megawatt Super-Phénix, which costs about twice as much as a French LWR plant of the same size. FBR advocates expect that cost-saving design changes, a relaxation of safety requirements and series production will help reduce construction costs. However, experience with the LWR suggests that it is not certain that cost savings through learning and series production will be quickly achieved. LWR costs have increased dramatically over the past decade. The effects of learning and series production have been cancelled out by other factors such as changes in licensing requirements, increasing quality assurance and control, and schedule slippages.

Estimated fuel-cycle costs for the SNR-300 are so high that the plant would not be commercially competitive even with nil investment costs. According to a forecast in 1973 by the utilities owning and operating the plant, operating costs will be such that the net returns from the sale of electricity are just about enough to pay usual depreciation and interest on a capital investment of DM 100 million. This is less than the amount to be invested in the reactor core (fabrication and plutonium), which for the SNR-300 is paid for by the state, but in commercial operation would have to be financed as a fixed cost by the utilities.

Over the past few years, the cost estimates for the SNR-300 fuel-cycle have become even more unfavourable. Fuel-fabrication costs have doubled in constant prices, while the fabrication costs of uranium fuel for LWRs have decreased in real terms. Thus the fuel-fabrication for the SNR-300 is now estimated to cost about thirty times more than for LWR fuel. The original estimate for reprocessing excluded the capital cost of the reprocessing facility.

It was based on the assumption that the fuel of the SNR-300 would be reprocessed in the West German WAK plant, but included no allowance for the construction cost of this facility or for the costs of its adaptation for FBR fuel. When exploring the possibility of reprocessing the SNR-300 fuel in foreign reprocessing facilities, the utility operating the SNR-300 received quotes that indicated much higher costs.

The dismally-high cost estimates for the SNR-300 are partly the result of the small scale on which the fuel cycle for this plant operates. But they also reflect soaring cost estimates for reprocessing of spent fuel and for fabrication of plutonium fuel in general. Because of its toxicity, plutonium fuel has to be fabricated in special plants. These plants can also fabricate plutonium fuel for LWRs. But at present they are still at a pilot or prototype scale.

Reprocessing facilities for LWR or advanced gas-cooled reactor (AGR) fuel can accommodate a few FBR fuel elements if these are reprocessed together with a larger number of LWR or AGR fuel elements. As long as the FBR capacity in operation is small compared with that of the LWRs or AGRs installed, this may be sufficient. Because FBR fuel has a higher plutonium content, generates more decay heat and contains more radioactive fission products than AGR or LWR fuel, the large-scale use of FBRs will, however, require either the modification of reprocessing facilities which were designed for AGR or LWR fuel or the construction of special facilities for FBR fuel.

Over the past decade, cost estimates for reprocessing of spent fuel and plutonium recycle have increased to a level that makes such operations commercially unattractive for LWRs at present uranium prices. The uncertainty persisting in these cost estimates is highlighted by the fact that present contracts with British and French reprocessors have basically a cost plus fixed-fee price. The cost of reprocessing will be known with some certainty only after the French and British have begun operating their large reprocessing facilities for oxide fuel now being constructed and when plutonium recycling is possible within a truly commercial framework.

Even then, however, some cost uncertainties will remain for the FBR fuel-cycle, because its technology differs from the plutonium fuel-cycle for LWRs. One such difference concerns cooling time. If spent fuel is stored before reprocessing, its decay heat and radioactivity decreases, so that reprocessing can cost less. The West German reprocessing plant for LWR fuel planned for a site in Hessen is intended for fuel with a cooling time of seven years. Shorter cooling times are essential for FBRs, to make effective use of their potential to breed new fuel and to reduce the financial charges on the fuel bound up in the fuel cycle. It may be technically possible to achieve the desired short cooling time, but it is not yet known what

engineering efforts will be necessary and what impact this will have on reprocessing cost. There is a real possibility that these economic uncertainties may come out unfavourably and that a very large increase in the price of uranium may be required to make the electricity costs of the FBR competitive with those of the LWR. This would delay FBR commercialization by several decades or more. In such an event construction and operation of a demonstration plant would require a large subsidy while the demonstrated technology would become obsolete before a larger number of FBRs are constructed.

Time-risk trade-off

Construction of a large FBR demonstration plant in the next ten years or so thus reduces only one of several key uncertainties. It will tell us nothing about the future growth of nuclear power or the availability of uranium; and it decreases only a little the uncertainty about fuel fabrication and reprocessing costs, as these services then will still have to be operated on a small scale.

On the other hand, no matter whether a large FBR demonstration plant is constructed or not, every year that passes gives us more information on future demand for nuclear power and the availability of uranium. And if we wait until rising uranium prices make plutonium recycling a commercially viable operation for LWRs, we shall have a much better basis to assess the economics of the FBR fuel cycle. Yet this may take a very long time if long-term storage of spent fuel proves environmentally as acceptable as fuel reprocessing, and the near-term future of nuclear power is based on LWRs operated in a once-through mode without reprocessing. But the rewards of waiting will be great in terms of reduced economic uncertainties.

As the fuel-cycle facilities of the FBR build on technologies of the LWR fuel cycle, there is a clear case of a time-risk trade-off. Constructing an FBR demonstration plant in the next ten years or so will involve much higher technical and commercial risks than doing the same thing later. And the analysis of uranium supply and demand suggests that there is no commensurable benefit to the public from taking the high risk now rather than taking the much lower risk later. If an FBR demonstration plant has higher costs than utilities are willing to pay, and if risk-sharing by the government is not sufficient to bring the commercial risk down to a level acceptable to the utilities, subsidies from the taxpayers' purse would be used only to take an unnecessary risk. In such a situation, the short-term calculations of utilities are likely to lead to a decision that is consistent with the public's long-term interest.

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NEWS AND VIEWS

Secondary structure and evolution of ribosomal RNA

from Richard Brimacombe

THE DNA sequencing boom has had an enormous impact on many areas of biochemical research, and the ribosome field is no exception. A series of complete rDNA sequences is now available, and this has led not only to the construction of convincing secondary structure models for the corresponding rRNA molecules, but also to the realization that these secondary structures, as well as significant regions in the primary sequences, have been conserved to a remarkable extent throughout evolution.

The large ribosomal RNA molecules vary considerably in size from one organism to another. At the lower end of the scale are the small mammalian mitochondrial species, with rRNA molecules of 12S and 16S in the small and large subunits (approximately 950 and 1,550 nucleotides in length, respectively). In the middle are the ribosomes from bacteria, together with chloroplasts and other types of mitochondria, containing rRNA molecules of 16S and 23S (~1,550 and 3,000 nucleotides) and at the upper end of the scale are the eukaryotic cytoplasmic ribosomes with rRNA molecules of 18S and 26–28S (~1,800 and up to 4,000 nucleotides, respectively).

Sequencing

The first rDNA sequence, that of 16S rDNA from *Escherichia coli*, was completed only three years ago¹. Now the list of available sequences includes the 23S rDNA from *E. coli*², the 16S and 23S rDNA from maize chloroplasts³, the 12S and 16S rDNA from human mitochondria⁴ and mouse mitochondria⁵, the 15S rDNA from yeast mitochondria⁶, and the 18S rDNA from yeast⁷ and *Xenopus laevis*⁸. Further sequences were reported at the recent EMBO Symposium on Ribosomes*, including the 23S rDNA from *Bacillus stearothermophilus* (Noller, Santa Cruz), 15S rDNA from *Aspergillus nidulans* mitochondria (Köchel, Göttingen), and the 26S rDNA from yeast (Planta, Amsterdam); the 28S rDNA from *Xenopus* is well on the way to completion (Gerbi, Providence). Partial sequences from a host of other organisms have also been described, and in addition the complete 16S molecule from *Proteus vulgaris* has been

worked out by direct RNA sequencing methods⁹. In short, we now have examples of ribosomal RNA or DNA sequences from all the principal categories mentioned above, with the notable omission of an archaeobacterial map.

Secondary structure

In order to get from primary sequence to secondary structure, sequence comparisons have been combined with various experimental approaches based on data derived from *E. coli* 16S and 23S rRNA. Three groups, in California, Strasbourg and Berlin, have been principally concerned in these studies, and each group has independently constructed models for the secondary structure of the ribosomal RNA from both subunits. The experimental approaches have been (1) the analysis of sites of chemical modification by single-strand specific reagents (California), (2) the analysis of sites of clipping by single- or double-strand specific ribonucleases (Strasbourg), and (3) the isolation and analysis of short base-paired fragments of the RNA, together with the determination of sites of intra-RNA cross-linking (Berlin).

The various possible secondary structural features suggested by these experimental data can then be tested by sequence comparisons, in order to derive the correct structure. This sequence comparison approach, which was successfully applied some time ago to the case of 5S rRNA by Fox and Woese¹⁰, is in essence very simple. If two very similar but not identical sequences from different organisms are compared, then a base change in one strand of a putative double-helical region must be compensated by a complementary base change in the other strand. Thus, an A-U base pair in one species could become a G-C pair at the corresponding positions in the second species, and so on. If the base changes between the sequences do not compensate each other in this way, then the implication is that the proposed element of secondary structure is incorrect. The secondary structure models for 16S and 23S rRNA have been successively refined by this approach, the latest versions (for the small subunit) being by Noller and Woese¹¹, Stiegler *et al.*¹² and Zwieb *et al.*¹³, for the three groups, respectively. In the case of the large subunit, the Berlin and

Strasbourg groups recently published structures almost simultaneously^{14,15}, whereas the model from the Californian group is still in the final stages of preparation.

The secondary structure models for both subunits are in about 80 per cent agreement with one another. Although there are still some important differences, the main features, that is, the 'long-range interactions' between RNA segments widely separated in the primary sequence and which serve to divide the molecules into well defined structural domains, are firmly established. It is also clear that all molecules in the same size class (16S or 23S rRNA) can be arranged in almost identical secondary structures, and the extent of primary sequence homology within this class is very high; maize chloroplast rRNA is, for example, 70 per cent homologous with *E. coli* rRNA, and if the two 23S molecules from these species are compared, then over 450 compensating base changes can be found between the proposed secondary structures.

Size difference

More exciting, however, is the finding that the structural comparisons can be continued outside the 16S and 23S rRNA size classes both to smaller and to larger rRNAs. The Berlin and Strasbourg publications cited above show that the small mammalian mitochondrial rRNA molecules (12S and 16S) contain many elements of secondary structure precisely equivalent to their 16S and 23S counterparts. Reduction in size is achieved by simple amputation of secondary structural loops, or by erosion of a whole domain. Recognition and location of secondary structural features is made easier by searching for stretches of conserved primary sequence, which tend to be — but are by no means exclusively — in single-stranded regions. There are, however, some interesting anomalies, where apparently significant stretches of homology have 'leap-frogged' each other in the different sequences. Precisely the same situation pertains if one makes a comparison with the longer eukaryotic

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*Held in Heidelberg from 27 September to 2 October, 1981.

cytoplasmic rRNA molecules (see also ref. 16). The same secondary structural features are conserved, and the extra sequences in the eukaryotic molecules are accommodated in precisely those regions which are shortened in the case of the small mitochondrial species. Again, there is striking conservation of primary sequence in both single- and double-stranded regions. The secondary structure comparisons also confirm the suggestions (based on primary sequence homology) that eukaryotic 5.8S rRNA is equivalent to the 5' end of prokaryotic 23S rRNA¹⁷, and

that the 4.5S rRNA from chloroplasts is correspondingly equivalent to the 3' end of the bacterial 23S rRNA¹⁸.

One should not be too surprised by these findings; after all, both tRNA and 5S rRNA have been highly conserved throughout evolution. What is important is that the extension of the conservation to the large ribosomal RNA molecules of all classes has now been clearly demonstrated, and that this conservation will *per se* be a powerful tool for testing the validity of any new structural or functional proposals in the future. □

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On Antarctic glaciology: Ice sheets. . .

from J. Weertman

AN international symposium on Antarctic glaciology* was held this autumn at Ohio State University. It was only the third meeting on this topic in over twenty years and it might be supposed that progress in this field moves at the proverbial speed of a creeping glacier. Actually, the pace of research has accelerated greatly in keeping, perhaps, with the recent discovery that the major glaciers in Antarctica, namely the great ice streams, move with impressive velocities.

Results were reported from research projects carried out in virtually all parts of the Antarctic continent, in oceanic islands

near the continent, and on the sea ice that surrounds it. The topography of the Antarctic ice sheets has been partially surveyed from satellites, with an elevation error of about 1.5 m, and the ice sheets have been probed by deep drilling (most recently by a 900 m core hole at Dome C in East Antarctica), by seismic sounding and by radar sounding. Data were obtained that describe in an increasingly quantitative manner the present ice mass and some of its past history and offer clues to its future behaviour.

The most interesting problem that remains to be solved in Antarctic

glaciology is whether or not the West Antarctic Ice Sheet (WAIS), as well as the East Antarctic Ice Sheet, is stable. It is suspected that it is inherently unstable because its base is far below sea level for most of its area. A related question is whether the expected increase in atmospheric CO₂ and the climatic warming it may produce will propel the ice sheet to its destruction.

Stability

The symposium opened with sessions addressed to the problem of ice sheet stability. Because most of the ice of the WAIS is drained through ice streams, the latter and their relationship to stability received much attention. One way to find out whether the WAIS is growing, is slowly disintegrating or is in a steady state is to see if the grounding lines where they meet ice shelves are stationary. British Antarctic Survey (BAS) scientists reported the first ever measurement of the position of an ice stream (Rutford Ice Stream) grounding line made with sufficient precision to determine within the next few years if it is stationary or not. We should not have to wait too long to learn whether the WAIS over the next century is benign.

Ohio State and Grenoble Universities glaciologists used indirect evidence of the volume of air in ice from the Byrd Station bore hole to conclude that the WAIS has been rather stable during past major climate and glacial changes. It has been stressed by University of Maine researchers that the Pine Island Glacier is where the WAIS may collapse but studies by BAS glaciologists have found no evidence of instability — although their findings cannot rule out its existence. The last 100 km of the Pine Island Glacier were found to be afloat and to be essentially a one-dimensional ice shelf. The existence of this ice shelf separates the calving front from the grounding line and probably increases stability.

Carbon dioxide

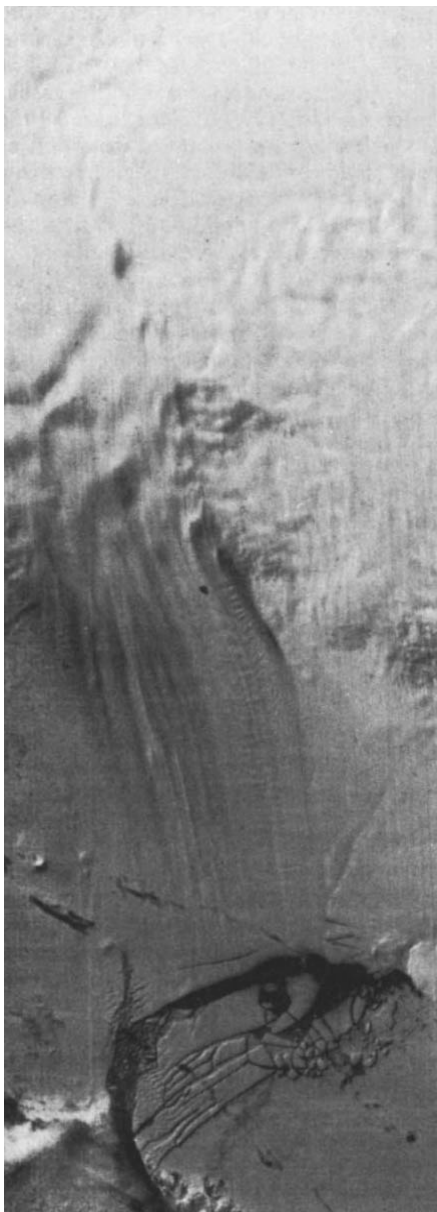
Whether a carbon dioxide build-up will make the Antarctic ice more or less stable is not certain. One definite piece of evidence was reported by University of Bern geochemists. The data, reconfirming their earlier work as well as that obtained at Grenoble, show that the CO₂ content of the atmosphere during the last ice age was lower than it is now. The measurements were made on entrapped air within ice cores brought up from deep within the ice sheets but whether the low CO₂ content is the cause, or is the effect, of an ice age is still anyone's guess. If a low CO₂ content and a large ice volume go hand-in-hand, then it is possible that a high CO₂ level could cause at least the West Antarctic ice to decrease in volume. A process that is likely to produce

Field camp on the Rutford Ice Stream with the Vinson Massif in the background.



Picture: J. Walton, British Antarctic Survey

*The Third International Symposium on Antarctic Glaciology was held at Ohio State University, Columbus, Ohio, 7-12 September 1981. The proceedings will be published as a volume in the *Annals of Glaciology*.



A NASA Landsat picture of the Pine Island Glacier. At the top of the picture the glacier ice is more than 2,000 metres thick but this falls to 500 metres where icebergs are calving off into Pine Island Bay at the bottom of the picture.

a reduction in ice volume is an increase in the rate of calving which would eventually lead to the destruction of the ice shelves and of the ice sheet they fringe.

Warming effect

The calving process is not well understood but a big advance was reported by University of Maine scientists who used rather detailed computer modelling calculations to study the process. We are still a long way though from predicting what would be the effect of, say, a 1°C warming produced by a CO₂ increase on the rate at which icebergs are created in Antarctica. □

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SINCE THE 1960s, when it was recognized that a chronological history of the composition of the atmosphere is contained in the Antarctic ice sheets, ice drilling has featured strongly in most national programmes. Analysis of ice cores is now making it possible to reconstruct variations in past climate, global volcanic activity and atmospheric pollution. The symposium provided an opportunity to review progress in these areas.

Particularly dramatic has been the evidence from deep ice cores of changes associated with the transition from the Wisconsin (Würm) glaciation to the Holocene. Lorius (Laboratoire de Glaciologie, Grenoble) discussed new data from the 905 m Dome C core which support earlier reports by Thompson (Institute of Polar Studies, Ohio State University) from this and the Byrd core that the number of microparticles increased by a factor of eight, relative to Holocene values, during the last glaciation.

But to what extent is the inverse relationship between increased particle load and temperature causal? Lorius

Glaciologie, Grenoble) has detected the influence on sulphate levels of several major volcanic events in both recent firn and in ancient ice back to 30,000 years BP. The input of sulphate from volcanic sources is only significant during the three to four years following an eruption. Such studies will provide an accurate record of volcanic activity over periods of tens of thousands of years and should also provide a powerful method for dating ice cores.

However, there is still a substantial input of sulphate in volcanically quiet periods which cannot be accounted for by input of marine salts. Delmas supports the idea that it originates in part from the oxidation of biogenic gaseous sulphur compounds, such as DMS, at the ocean surface. The aerosol may then be transported to the polar plateau through the upper troposphere. On the other hand Herron (State University of New York, Buffalo) has found clear seasonal variations of sulphate with summer maxima which do not correlate with marine chloride. Thus he believes a non-marine gaseous sulphur compound is the most likely source, with

. . . and ice cores

from David A. Peel

argues that a global increase in arid areas during the last glaciation combined with a fifty per cent increase in wind speed associated with a strengthened meridional circulation would be sufficient to account for the enhanced microparticle load. In the sections of core analysed by his team he could find no evidence for increased volcanic activity in the glacial period either by examination of the larger particles, or in the levels of gas-derived sulphate. However, Thompson has clearly identified bands of large (60–80 µm) volcanic glass fragments in other segments of the same core and found they occurred with greater frequency in the Wisconsin. Clearly, a means must now be sought to distinguish whether dust in the smaller and more abundant size ranges is volcanic or continental in origin. Millar (Scott Polar Research Institute, Cambridge) gave evidence that radio-echo sounding can detect the acid layers associated with volcanism and provides a means for deducing broad changes in the level of volcanic activity over periods of tens of thousands of years. This type of study could, in the future, rapidly extend knowledge previously based on the much slower analysis of ice cores.

The major impurity in Antarctic ice is sulphate yet the mechanism by which it reaches the Antarctic ice sheet is largely unexplained. Application of new methods has allowed much more detailed sampling from a wider area than before. In the Dome C ice core Delmas (Laboratoire de photooxidation of the gaseous precursor

photooxidation of the gaseous precursor playing a significant role. A clear inverse relationship between snow accumulation rate and sulphate concentration at sites in both Greenland and Antarctica further indicated dilution by snowfall of aerosol derived from a gaseous precursor in the upper troposphere or lower stratosphere.

Nitrates

Nitrates in snow are presenting greater difficulties. Zeller has made extensive measurements on 100 m cores and shallow pits at South Pole and Vostok station which support his earlier evidence that much of the NO₃-N in Antarctic snow is derived by auroral fixation of NO_x and follows an 11 year cycle in solar activity. Elevated NO₃-N levels coincided with years of solar flare activity peaks which are associated with high auroral activity. Pronounced seasonal variations with maxima in winter months could be due to summer photochemical destruction of much of the NO_x. The broad, longer term changes at the South Pole and Vostok were similar with prolonged, low NO₃-N concentrations at the time of the Maunder minimum (1617–1710).

In contrast, Herron, who also included NO₃ measurements from the South Pole in a detailed investigation of the anion content of snow, although agreeing with the mean NO₃ values reported by Zeller,

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observed much less variance in concentration even with a greater sampling frequency and could not detect any deviation during the period of the Maunder minimum or any of the spikes which Zeller attributes to solar flare or supernovae events. Moreover, he detected a seasonal variation with summer maxima in parallel with variations in sulphate. Delmas also found that nitrate concentrations in the

Dome C core have been relatively constant over the last 30,000 years. To complicate the picture even further, in a detailed profile at James Ross Island he found virtually no seasonal variability. In view of the potentially great value in using nitrate to trace past solar fluctuations the conference agreed that high priority should be given to resolving these seemingly incompatible data series. □

A virus associated with human adult T-cell leukaemia

from Robin Weiss

RECENT virological, serological and epidemiological evidence strongly indicates that a newly discovered retrovirus is the aetiological agent of certain types of adult T-cell lymphoma/leukaemia. The virological evidence first came from R.C. Gallo's laboratory at the US National Cancer Institute¹ where the retrovirus was isolated from a T-lymphoma cell line established in culture from a patient with mycosis fungoides. In this issue of *Nature* Poiesz *et al.* (see p.268) report the isolation of a similar virus from a patient with Sézary syndrome, while Kalyanaraman *et al.* (see p.271) show that the two patients' sera (and that of a patient's spouse) contain antibodies which specifically react with the major core protein of the virus.

Mycosis fungoides and Sézary syndrome are clinical variants of cutaneous T-cell lymphoma and leukaemia, respectively, that occur as rare diseases in adults². The retrovirus particles produced by these tumours are not evident until the tumour cells are grown in culture, although a second biopsy from the first patient released virus within 24 h of its being placed in culture. Until 5 years ago it was very difficult to maintain normal or malignant T cells in culture; however, a specific T-cell growth factor (TCGF) discovered in Gallo's laboratory³ enables T cells to proliferate *in vitro* for long periods, and this aided the detection of the virus.

Properties of HTLV

The virus, called HTLV (for human T-lymphoma virus), seems to be quite distinct from the numerous types of animal retroviruses previously described. By its morphology and the molecular weight of virion proteins, HTLV most closely resembles bovine leukaemia virus, which causes lymphoma in cattle. However, the human and bovine viruses seem unrelated antigenically or by nucleic acid homology.

Following Gallo's report of virus production from T-lymphoma cultures, an

independent virus isolate has been made in Japan⁴ from a previously established malignant T-cell line⁵. Now Miyoshi and his colleagues, in a paper shortly to be published in *Nature*, claim that co-cultivation of the T-leukaemia cells with umbilical cord leukocytes leads to the transformation of the cord leukocytes into a TCGF-independent cell line producing retrovirus particles. This is the first experimental evidence for infectious transmission and cell transformation with HTLV.

Origin of HTLV

Gallo's laboratory has not succeeded in transforming animal or human cell lines with HTLV, but they have not tested cord lymphocytes nor used co-cultivation. They think that T lymphocytes from some relatives of patients may support the replication of HTLV. At a recent meeting of the Leukaemia Research Fund in England, Gallo reported individual isolates of HTLV from four cutaneous T-lymphoma patients. From one patient an Epstein-Barr virus-positive B-lymphoblast line was also developed: this line was negative for HTLV, suggesting that HTLV is specifically associated with the tumour cells. Serological tests on some hundreds of American normal individuals or patients with diseases other than T-cell malignancies have been negative for antibodies reacting with the HTLV p24 core protein. By no means all T-cell leukaemia patients are positive either; the virus appears to be associated with a particularly malignant, fast growing form of tumour. Gallo thinks that all four patients (three black and one white) from whom HTLV has been isolated have Caribbean connections and that the virus may be more commonly found there.

The most striking epidemiological studies, however, come from Japan. There is a relatively high incidence of patients in major Japanese cities presenting an aggressive form of adult T-cell leukaemia. Uchiyama *et al.*⁶ noted that 13 out of 16 patients with this form of disease were born and grew up on the west coast of Kyushu,

Japan's extreme south-west island. A more recent study⁷ of 272 T-cell leukaemia cases shows a remarkable clustering of places of birth, in the Kagoshima and Nagasaki prefectures of Kyushu. Sera from all the leukaemia patients tested and from 25% of healthy adults sampled react positively with Miyoshi's virus isolate⁴. Through Yohei Ito, Gallo has found that Japanese sera also recognize his viral p24 antigen.

Several viruses are implicated as aetiological factors in human malignancies; Epstein-Barr virus in Burkitt's lymphoma and nasopharyngeal carcinoma are the classic examples. The association of hepatitis B virus infection with liver cancer has become apparent although it is curious that this virus does not even merit passing mention in two major compendia on tumour viruses published as recently as 1980^{8,9}. Cancer of the uterine cervix may well have an infectious agent as an aetiological factor, for which herpes simplex and papilloma viruses are under suspicion. During the 1970s claims for isolating human retroviruses have perhaps been made too frequently and prematurely. While some proved to be animal viruses acquired during laboratory procedures, others remain enigmatic, such as the virus-like particles seen in full-term placenta¹⁰. Four laboratories — Bentvelzen's in Holland, Gallo's at the National Institutes of Health, Kaplan's at Stanford and Kirsten's in Chicago — have independently isolated from human tissues retroviruses related to those of gibbons and baboons. These isolates are generally dismissed as laboratory contaminations. I am reluctant to disclaim their human provenance so lightly, although admittedly human antibodies which were thought to bind to these viruses specifically now appear to recognize heterophile carbohydrate groups present on the viral glycoproteins^{11,12}.

Role in malignancy

The striking features of the T-cell leukaemia virus are its unique biochemical properties, its association with a particular subset of T-cell lymphoma/leukaemias and the geographical clustering of that disease in Japan. The discovery of this new virus followed the successful development of culture methods for T cells. The epidemiological findings would not have been possible without careful classification of leukaemias into B- and T-cell classes and further subclasses. A fascinating and important story is unfolding on the viral aetiology of a human malignancy. □

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Progress on accretion disks

from J. Craig Wheeler

ASTROPHYSICISTS are wrestling with the study of a new kind of star, the flat two-dimensional configurations known as accretion disks. Accretion disks exist in a variety of contexts where mass is deposited to swirl around a compact star such as a white dwarf or a neutron star. They are suspected to play a part in more exotic situations, like active galactic nuclei and quasars, where the central object is imagined to be a supermassive black hole. The study of these objects is still in its infancy, analogous to the early days of Eddington when stars were modelled using elementary scaling laws without benefit of knowledge of the nuclear processes which powered the stars. Similarly, the power by which accretion disks radiate is suspected to come from a form of turbulent friction, the basic physics of which is known only at the crudest level. Most of the current literature on accretion disks is couched in terms of parametrized scaling laws which avoid direct confrontation with our most basic levels of ignorance. The letter by Abramowicz in this issue of *Nature* (p.235) adds needed rigor to our understanding of the structure and evolution of accretion disks while exploring an instability in the disk structure which has plagued earlier models.

Accretion disks were first defined as astronomical entities in the context of cataclysmic variables. In these systems, which include novae, matter from the outer layers of an ordinary star is attracted by the gravitational influence of a nearby orbiting white dwarf star. The matter lost from the ordinary star cannot strike the surface of the tiny white dwarf directly, but settles into nearly keplerian orbit. The viscosity in the disk causes heating and radiation and a slow spiralling of the material onto the surface of the white dwarf.

The rapid advances made in X-ray astronomy in the past decade have identified similar systems in which the accretion disk whirls about a neutron star rather than a white dwarf. The inner reaches of the accretion disk extended deeply into the gravitational potential of the neutron star where very rapid motion is the rule. The energy released by friction and the actual raining of the material from the disk onto the surface of the neutron star is so great that the radiation comes off in a powerful flood of X rays. In at least one binary system explored by X-ray astronomers, Cygnus X-1, the object in the centre of the accretion disk is thought by many to be a black hole, the ultimate form of collapsed matter.

Complete understanding of the out-

bursts of cataclysmic variables and X-ray production by neutron stars demands knowledge of the associated accretion disks. Through such study may also come deeper insight into phenomena such as active galaxies where matter from millions of stars may gather around a gigantic black hole in the galactic centre.

Previous work has shown that portions of accretion disks are unstable, leading to the clumping of matter into rings or bloating into a fat hot doughnut, in contradiction to assumptions that the disks are geometrically thin. However, there is no observational evidence that accretion disks do, in fact, suffer from these instabilities. Abramowicz argues that these instabilities may not occur in reality, and he shows that added gravitational effects due to general relativity alter the potential compared with the standard newtonian case in such a way that the instabilities are removed.

Ironically, this work may have most direct application in the case of accretion disks around supermassive black holes where observational confirmation of the

basic picture is weakest. Abramowicz argues that the major inner radiating portions of such a supermassive disk will be stabilized in this case. The relativistic effects of which he speaks should also apply to putative stellar mass black holes, as in the case of Cygnus X-1, but this system is known to undergo some transitions in its radiative properties which have been discussed in terms of disk instabilities. For systems with white dwarfs and neutron stars the surface of the star or the presence of a surrounding magnetosphere may dominate the inner portions of the disk and overwhelm the relativistic effects described by Abramowicz. Even in the case of supermassive black holes there is speculation that the inner region consists of a nearly spherical, differentially rotating configuration, so that the basic assumptions underlying a thin disk analysis do not apply.

This discussion serves to illustrate the complexity of the phenomena known collectively as accretion disks. Progress towards understanding them will involve defining and solving restricted problems just as was done, and continues to be done, for ordinary spherical stars. The work by Abramowicz gives a new valuable example of the care which must be taken before reaching definitive conclusions regarding accretion disks. □

Geomechanics in the laboratory

from Neville G.W. Cook

THE increasing use of the ground beneath us, whether in mining activities, underground construction or in the disposal of toxic wastes, requires, as does an understanding of earthquakes, a much deeper knowledge of the behaviour and properties of rocks than has sufficed in the past. To understand the behaviour and properties of rock masses, *in situ* tests are possible but they are costly and time consuming¹⁻³. As became clear at a recent conference (see *Geophysical Research Letters* 8, 1981 for the proceedings) there is a need for laboratory tests on a scale of the same order as that of the *in situ* tests, both to avoid many of the restrictions of *in situ* tests and to bridge the gap between them and usual laboratory tests.

The most common kind of laboratory test on rock is the 'triaxial' test first introduced in 1911 (see ref.4). Right circular cylinders of rock, usually a few centimetres in diameter and several times greater in length, are subjected to axial compression between the platens of a mechanical press and an equal radial compression, applied through an impermeable membrane by a confining fluid pressure.

There are fundamental differences in the problems faced by laboratory testing in increasing deformation leads to decreasing

engineering mechanics and in geomechanics. In engineering mechanics, man-made materials are generally used and laboratory specimens can be expected to have similar properties to those of structural members made of the same material. Such materials are relatively simple in composition and structure compared with the composition and structure of most rocks. Again, laboratory test specimens of man-made materials are usually within an order of magnitude of the size of structural members whereas the dimensions of excavations and geological features can be many orders of magnitude greater than those of the rock specimens.

A further problem is that systems comprising conventional, soft testing machines and specimens of brittle rock become unstable at or beyond the peak of the stress strain curve⁵. Until the advent of stiff or servo-controlled testing machines, it was impossible to study the 'work-softening' deformation of rock, where increasing deformation leads to decreasing

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resistance to deformation. Stable, work-softening deformation is of great importance in many practical problems, such as the stability of underground excavations and aseismic movement along faults. A final difference is that it is possible to subject specimens of man-made materials to loads similar to those they will experience in use, while small laboratory tests cannot reproduce the effects of stress concentrations in metres to kilometres in a rock mass subjected to a poorly understood state of stress.

Despite these limitations, the triaxial laboratory test has been of immense value in providing scientists and engineers with an understanding of the behaviour and properties of rock. As examples can be cited the transition from brittle to ductile behaviour as a function of confining stress and temperature, the effects of fluid pressure, and the phenomena of dilatation and brittle cracking in compression^{6,7}.

The behaviour and properties of most rock masses *in situ* differ from those measured in conventional laboratory tests on specimens of the same rock. The reasons for these differences are not well understood but it is likely^{8,9} that size is one

of the factors involved. There are likely to be many others, such as variations in geological composition and structure, on all scales.

At present, three large-scale laboratory test facilities using specimens of rock with dimensions of the order of a metre are in use in the United States. The three facilities have each been designed for a specific purpose — the Lawrence Berkeley Laboratory triaxial apparatus for testing the mechanical and hydraulic properties of jointed rock at low stresses¹⁰, the US Geological Survey's biaxial frame for testing simulated faults in large blocks for rock¹¹, and the Terra Tek Drilling Research Laboratory.

The proceedings of the workshop made it clear that large-scale laboratory testing is likely to be much more costly than is conventional laboratory testing, but such a facility will answer fundamental questions in rock mechanics. Comprehensive measurements of phenomena occurring inside a rock specimen, such as the location of sources of acoustic emission and fracture initiation and propagation, could be made under a wide range of controlled conditions of stress or strain, pore fluid

pressure and temperature.

It was pointed out that the simplicity of the triaxial test is deceptive; it, in fact, involves homogeneous axial strain and homogeneous radial stresses. A large-scale triaxial high pressure and temperature cell may be excessively costly and potentially hazardous. A successful large-scale laboratory testing facility is likely to have to be different in concept and design from a conventional triaxial cell. □

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Morphological stasis and developmental constraint: real problems for neo-Darwinism

from Peter G. Williamson

A recent *News and Views* article¹ commented on a paper of mine² which summarized a morphometric analysis of mollusc lineages from a Cenozoic sequence in North Kenya. I had made three key points: (1) all lineages exhibit morphological stasis for very long periods of time (3–5 Myr), (2) evolutionary change in each lineage is concentrated in relatively rapid speciation events (occurring over 5,000 to 50,000 years) and (3) the speciation events are accompanied by pronounced developmental instability in the transitional populations.

The first two points indicate that these lineages conform to the 'punctuated equilibrium' model for evolutionary change and the third is significant because the unusually complete Turkana Basin sequence offers the first fine-scale palaeontological documentation of the speciation process.

The *News and Views* article considers only the second of these three points, making the uncontested observation that geneticists have succeeded in producing significant phenotypic changes in many populations over periods considerably shorter than those required for speciation events documented in the Turkana Basin mollusc sequence. The question of *rapidity* of speciation events is addressed but no attention is paid to the problems either of long-term morphological stasis or of

developmental instability during speciation. Such comments are the standard but largely tangential criticisms advanced by many evolutionists against the punctuational model. The possibility of rapid change is freely admitted, but the significance of stasis, and the implications of developmental constraint for evolutionary process, are ignored. Since the critique (and others recently published)^{3–5} seem to miss the most important issues raised by punctuated equilibrium theory in general, and my own work in particular, some comments seem in order.

As Gould and Eldredge⁶ and many others have repeatedly pointed out, punctuated equilibrium is a theory about the *deployment of speciation in time*. It holds that many, perhaps most, metazoan fossil sequences show a characteristic pattern of morphological change through time: new species enter the record abruptly (in geological time), and persist with little significant change until extinction. Significant evolutionary change is, therefore, concentrated at speciation events.

For reasons that elude many of us, punctuated equilibrium, a theory of evolutionary tempo, has been conflated

with Goldschmidtian macromutation, a theory of evolutionary mode. Thus, in the critique of my paper, I am lumped with Goldschmidt (and Cuvier of all people) as disciples of some peculiar (and non-existent) non-Darwinian evolutionary school. But punctuated equilibrium is compatible with much current neo-Darwinian thought. Eldredge and Gould⁷, in their original formulation, relied upon the most orthodox version of Mayr's theory of allopatric speciation via peripheral isolates to account for the 'punctuational' pattern of morphological change in the fossil record. They considered the abrupt appearance of new species and their subsequent stasis, as well as the lack of intermediates between such stable lineages, to be compatible with (indeed, to flow from) Mayr's model. Mechanisms for rapid speciation that have been proposed subsequently (for example, the various models for 'chromosomal' speciation) are compatible with but not required by punctuated equilibrium, a theory of evolutionary tempo that is agnostic about modes of speciation, so long as they yield (as most standard models of speciation do) the punctuational pattern when translated into geological time.

It is not news to punctuationalists that population geneticists can produce rapid phenotypic shifts in artificial selection regimes within a few generations —

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punctuationalists and conventional neo-Darwinians are in complete agreement on this point. Why, then, are many punctuationalists increasingly unhappy with conventional neo-Darwinian accounts of fundamental evolutionary process?

The principal problem is morphological stasis. A theory is only as good as its predictions, and conventional neo-Darwinism, which claims to be a comprehensive explanation of evolutionary process, has failed to predict the widespread long-term morphological stasis now recognized as one of the most striking aspects of the fossil record. The long-term morphological stasis noted by punctuationalists in the fossil record is clearly mirrored by the relative morphological uniformity of most widely distributed modern species. As Ernst Mayr, the foremost student of geographical variation has written⁸: '... it would ... seem important to stress the basic uniformity of most continuously distributed species ... The fact that (every taxonomist) ... can identify individuals of a species ... regardless of where in the range of the species they come from is further illustration of this phenomenon'. In a belated attempt to address the problem of morphological stasis, neo-Darwinists have invoked 'stabilising selection' (for example, Stebbins and Ayala⁵). But the wide range of environments presently exploited by extensively distributed but morphologically uniform modern species, and the long-term morphological stasis (up to 17 Myr) exhibited by many fossil lineages in fluctuating environments, strongly argues against the idea that simple stabilising selection is an adequate explanation for the phenomenon of morphological stasis. Accordingly, Mayr⁸ explicitly invokes some form of developmental homeostasis, rather than stabilising selection, to explain the range-wide morphological stability of most modern species.

In the original formulation of punctuated equilibrium theory, it was suggested that temporal stasis, like the geographic stability noted by Mayr, was largely the result of some form of developmental constraint or homeostasis. In the absence of a comprehensive genetics of development, the mechanism for such homeostasis is obscure. But if some form of developmental homeostasis is at the root of morphological stasis, speciation must, by definition, involve the dismantling of homeostatic mechanisms pre-existing in the parental stock. The principal argument in my paper is that when speciation events occur in the Turkana Basin mollusc sequence, they are invariably accompanied by major developmental instability (that is, just such a dismantling of developmental homeostasis). The idea that morphological stasis is primarily a result of developmental homeostasis, and that speciation must therefore involve the temporary dismantling of such a homeostatic system,

differs from most conventional neo-Darwinian assumptions about the way in which species arise. Most neo-Darwinists would agree with Darwin's statement that new species arise 'solely by accumulating slight, successive, favourable variations'. They believe that the intrapopulation micro-evolutionary changes observed in the *Drosophila* cage can be simply extrapolated into the differences between *Drosophila* species and Dipteran families. In this view of speciation, as Gould⁹ says, there is a 'seamless continuum' a 'smooth extrapolation ... from base substitution to the origin of higher taxa'. There is no suspicion here that the fundamental developmental constraints implied by long-term geographical and temporal stasis of species must be dismantled when new species arise. There is no suspicion that this radical reorganization of fundamental homeostatic mechanisms during speciation must involve a more radical overhaul of the phenotype than the steady 'march of metric means' seen in a *Drosophila* cage experiment.

Interestingly enough, the idea that disruption of developmental homeostasis, or constraints, is central to the speciation process is hardly new to the neo-Darwinian literature: Carson¹⁰ has postulated 'open' and 'closed' genetic systems, the former involved in the allel-shuffling of minor adaptive adjustments within species populations, and the latter involved in the more profound regulatory

and developmental changes during speciation. Levin¹¹ has pointed out the significance of developmental disruption and instability during the speciation process. But such suggestions have been largely ignored: the implied decoupling of micro-evolutionary change and macro-evolutionary phenomenon threatens the reductionist core of conventional neo-Darwinism.

The Turkana Basin sequence records a pattern of long-term stasis punctuated by rapid speciation accompanied by pronounced developmental instability. Jones suggests that this pattern requires no change in conventional views of the genetic mechanisms of the origin of species — despite the fact that this pattern is neither predicted by neo-Darwinism nor explicable in terms of its major tenets. Punctuationalists suggest that it is time for conventional neo-Darwinism to address the important issues of morphological stasis and developmental constraint. □

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Life on an oily wave

from A.J. Southward

AFTER three years of deliberation, the Royal Commission on Environmental Pollution has decided that chronic and accidental spills of petroleum do not pose a permanent threat to marine life comparable with the dangers inherent in heavy metals and radioactivity (*Nature* **293**, 692; 1981). This view, with which many scientists concur, is based on the assumption that petroleum is degraded by physical, chemical and biological breakdown, and thus does not have a cumulative effect. Nevertheless, there is still public concern about oil in the environment because it is so evident, whether fouling the shoreline or killing sea-birds. Scientists too are concerned about the extent to which marine communities are disturbed by oil, and how long they take to return to normal. In consequence, a meeting* was held recently to discuss the long term effects of oil pollution on marine populations, communities and

ecosystems. There was considerable disagreement about the importance of sub-lethal effects; the length of time needed for recovery; and whether the effects of pollution can be detected when natural communities show extensive short and long term fluctuations in abundance and species composition. However, some consensus was reached that planktonic and pelagic communities have been relatively untouched by oil pollution (J. Davenport, Marine Science Laboratories, University College of North Wales). This and evidence that even badly affected salt marsh communities can begin recovery after improvements to refinery effluents (B. Dicks, Orielton Field Centre, Pembroke) allowed cautious optimism about the future.

The first contribution set the scene by describing the origins and fates of the estimated six billion tons of oil that reaches

*Discussion meeting at the Royal Society, 28–29 October 1981, organized by Dr H.A. Cole & Professor R.B. Clark. A full report is to be published in *Phil. Trans. R. Soc. B*.

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the sea each year (K.J. Whittle, MAFF Torry Research Station, Aberdeen). None of the following contributors who described the biological effects of oil defined what they meant by 'long term' and it was left to the penultimate speaker, Sir Hans Kornberg, FRS (Chairman Emeritus of the Royal Commission on Pollution) to quantify this term when he referred to the preceding papers as discussing "serious short term effects". Evidently the Royal Commission must regard long term as periods greater than the 10 to 20 years mentioned during the meeting as the time needed for recovery by some ecosystems after oil spills. In contrast, most of the contributors evidently thought that 10 to 20 years was a long time in comparison with the severe mortalities that ensue a few hours or days after major and minor spills (J.H. Vandermeulen, Bedford Institute of Oceanography, Dartmouth, N.S.).

It was suggested that repeated oil spills on one coast, such as in Brittany since 1967, might put off final recovery of affected communities to the next century (A.J. Southward, Marine Biological Association, Plymouth), but this was thought too pessimistic by the chairman (R.B. Clark, University of Newcastle) who noted that communities and ecosystems were remarkably resilient. This appears to be true for sea-birds, which suffer the most distressing casualties seen by the public, but which are mostly long-lived species undergoing heavy natural mortality each year, when hundreds of thousands die compared with tens of thousands killed by oil (G.M. Dunnett, University of Aberdeen). As confirmed in the discussion (W.R.P. Bourne, University of Aberdeen), some species can make up for heavy mortality in colonies by accelerated maturation of the juveniles.

Marine fishes suffer much heavier

natural mortalities than birds during their life history, and are also subjected to heavy pressure from fisheries. Fish populations, and catches by fishermen, undergo extensive natural fluctuations which, without the records of fishery statistics, might be ascribed to pollution rather than to natural changes or overfishing (R. Jones, DAFS Marine Laboratory, Aberdeen). Other marine populations, including those on the seashore, undergo extensive natural fluctuations, and it may be difficult to detect pollution effects (J.R. Lewis, Wellcome Marine Laboratory, Robin Hood's Bay, Yorkshire). No general signs of oil-induced mortalities have been detected in commercial fish stocks (A.D. McIntyre, DAFS Marine Laboratory, Aberdeen), but tainting of the flesh may be evident after spills, and there is some suggestion of local inshore population changes in oysters and shrimp, related to chronic oil pollution. Other shellfish, especially *Mytilus*, are well known to be sensitive to low levels of petroleum hydrocarbons or other forms of sublethal environmental stress (B.L. Bayne, Institute for Marine Environmental Research, Plymouth), which can be detected as changes in metabolism and gonad development. It is therefore surprising that little general effect of natural oil seeps can be demonstrated in the *Mytilus californianus* community around Santa Barbara (Dale Straughan, Institute for Marine and Coastal Studies, University of Southern California, Los Angeles). Offshore petroleum operations in the Gulf of Mexico also appear to have had little effect on shell fisheries and the bottom fauna in general (J.M. Sharp, Gulf Universities Research Consortium, Houston), but signs of pollution-induced changes in the benthos are appearing close to a North Sea oil-rig (J.P. Hartley, Orielton Field

Centre, Pembroke).

Nevertheless, there is abundant experimental and circumstantial evidence that oil pollution is associated with histopathological lesions in fish and genetical abnormalities in pelagic eggs and larval stages (C.J. Sindermann, NOAA, Sandy Hook Laboratory, New Jersey). Such effects were seen in flatfish after the 'Amoco Cadiz' spill in Brittany (M. Conan, CNEXO, Brest), where long-lasting retention of oil in fine sediments may be delaying recovery of benthic animals and salt-marshes. It was thought that some salt-marshes, which were damaged by clean-up measures as well as oil, may take 20 years to recover. Comparable lengths of time have been suggested for tropical mangrove communities, which are very sensitive to oil damage (J. Baker, Orielton Field Centre, Pembroke). Both these types of community tend to be in places where oil spills are more likely.

The meeting demonstrated that there are long term chronic effects of oil, even if the evidence is partly circumstantial; but although damage from accidental spills can be severe and take many years to repair, the general effects are not as bad as once feared. It was felt that the tendency for committees to recommend further experiments on detection of effects of oil pollution should be resisted and replaced with ecological monitoring having well defined motives and objectives: there should be no hesitation in abandoning or reducing surveys where no ill effects were apparent. It is hoped that the recommendations of the Royal Commission will be acted upon by the Government, particularly those concerned with the prevention of oil spills, searches for clean-up techniques not dependent on chemicals and the setting-up of a central unit for co-ordinating action after spill. □



99 years ago

WEATHER FORECASTS

I have recently designed and patented "An improved floating vessel for automatically compressing air by the action of the waves of the sea, and also for the generation of electricity by the agency of this compressed air." This vessel is capable of being moored in 1000 fathoms, and can be connected with the shore by means of an insulated electric cable. Such a vessel moored in the mid-Atlantic in the usual track of the cyclones which approach these islands from the west, would be of immense advantage to the Meteorological Office in determining the velocity of advance and direction taken by these cyclonic centres. I purpose exhibiting a model and drawings of the vessel at the Winter Electric Exhibition, to be held at the Westminster Aquarium next month.

CHARLES W. HARDING

THE MAGNETIC STORM AND AURORA

The telegraphic system of this country has, since Friday morning last, been disturbed in a way that far exceeds anything of the kind that has ever happened before. Very powerful electric currents have been swaying backwards and forwards through the crust of the earth, taking all telegraphic circuits in their progress, and entirely stopping communication. Communication has been maintained only where it was possible to loop together two wires, so as to avoid the use of the earth altogether. The electric storm commenced on Thursday, but it reached its climax on Friday morning (November 17) between 10 and 11 a.m. The currents measured over 50 milliampères, which is five times greater than the ordinary working currents. They have repeated themselves at intervals ever since, but have scarcely attained such an intensity as on Friday morning.

Both the storm and the aurora seem to have extended to America; the Philadelphia correspondent of the *Times* telegraphs under date November 19:—

"The electrical storm which began to derange the telegraph wires on Friday last still continues, though with less intensity. It spread

through Canada and the greater part of the United States, as far west as Utah. The electricians say that the disturbance was unlike any heretofore known, acting upon the wires in strong waves, which produced constant changes in the polarity of the current. A magnificent aurora appeared on Friday night and was visible at all points, except where clouds obscured it. Cold weather, with snow, accompanied the storm in many places."

Mr W.H.M. Christie, the Astronomer Royal, writes —

In the evening, as soon as it was dark, a brilliant aurora was seen, commencing with a bright glow of red light extending from the north and west beyond the zenith, interspersed with pale green phosphorescent light and streamers. At 6h. 4m. a very brilliant streak of greenish light about 20° long appeared in the east-north-east, and, rising slowly, passed nearly along a parallel of declination, a little above the moon, disappearing at 6h. 5m. 59s. in the west, about two minutes after it was first seen. The whole aurora had faded away by about 7h., but it burst out again at 11h. 45m., when an auroral arch, with brilliant streamers reaching nearly to the zenith, was seen from north-north-east to north-west.

From *Nature* 27, 79 & 82, November 23, 1882.

REVIEW ARTICLE

λ Repressor and *cro*—components of an efficient molecular switch

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In a lysogen, most genes of phage λ are repressed; in response to a transient induction signal, they are efficiently switched on. The switch, which consists in part of a tripartite operator to which two regulatory proteins bind, depends not only on DNA-protein interactions, but also on effects transmitted from one DNA-bound protein to another. λ exemplifies a strategy that facilitates efficient switching between two physiological states in response to a transient signal.

THE genome of the temperate bacteriophage λ can exist in either of two states. Switching from one state (lysogeny) to the other (lytic growth) is induced with high efficiency by external agents such as UV light, and virtually all lysogens in a population can be induced synchronously by such treatments. The genes *cI* and *cro*, upon which lysogeny and lytic growth respectively depend, encode key components of the system. The protein product of each of these genes (repressor and *cro*) turns off the other gene. In a lysogen, repressor is made, but not *cro*; after the switch to the lytic cycle, *cro* but not repressor is synthesized (Fig. 1).

We have recently described the molecular mechanisms of action of repressor and *cro*¹. These two DNA-binding proteins recognize the same operator sites but have opposite physiological effects. The action of repressor is particularly elaborate: it regulates transcription both positively and negatively and, in doing so, engages in three different cooperative interactions. Why is the action of repressor so complex?

To explore this and related questions, we first review present knowledge of how λ repressor and *cro* function. We then consider studies, largely unpublished, of 434 and P22, two other temperate phages which have repressors, *cro* proteins and operators that differ in sequence from those of λ . We find that the general structures of the operators, the patterns of positive and negative control and the types of cooperativity shown by the repressor are similar in all three phages despite protein and DNA sequence differences and differences in detailed mechanism. We argue that these common features serve the same vital role for each of the three phages: they ensure stable lysogeny and allow efficient switching to lytic growth on induction. Thus, the lytic genes of the phage are very tightly repressed in the lysogenic state, yet they are efficiently and irreversibly switched on by a transient induction signal. Such a 'hairtrigger' response would be difficult to achieve with a repressor-operator system, such as that found in the lactose operon, that lacks the cooperative features of the λ system.

λ Repressor

Phage λ bears two operators to which repressor and *cro* bind. Here we consider mainly the right operator (O_R), the primary locus of the lysogen-lytic growth switch. This operator consists of three sites, each of which can bind either repressor or *cro*, but not both simultaneously. These operator sites control two divergent promoters, P_R and P_{RM} (see Fig. 2). The activity of each of these promoters depends on which of the three operator sites are occupied by repressor and/or *cro*. For example, at O_R a

prophage bears repressors bound mainly to sites O_{R1} and O_{R2} . In this state, repressor turns off transcription from P_R and turns on transcription from P_{RM} . As a consequence, the genes required for lytic growth (including *cro*) are repressed, while the gene required to maintain the lysogenic state (*cI*) is active. Repressor is a positive as well as a negative regulator: in its absence P_{RM} functions very inefficiently. The experiments that elucidated the effect on gene transcription of repressor or *cro* bound to each of the three sites in O_R are described elsewhere¹⁻⁷.

Figure 3a shows a molecular model of the topography of repressors at O_{R1} and O_{R2} and RNA polymerase at P_{RM} in a λ lysogen. Three sets of protein-protein interactions are implicit in the figure: (1) dimer formation by repressor; (2) interaction between repressor dimers bound to O_{R1} and O_{R2} ; and (3) interaction between a repressor dimer bound at O_{R2} and an RNA polymerase molecule at P_{RM} . We shall consider these three different cooperative interactions in turn.

Formation of dimers. λ Repressor monomers are in equilibrium with dimers, but only dimers bind tightly to the operator⁸⁻¹⁰. One dimer occupies one operator site. Repressor monomers (236 amino acids) consist of two structural domains, each encompassing ~100 amino acids, joined by a protease-sensitive connector¹¹. The amino-terminal domains recognize the operator sites and the carboxy-terminal domains provide the most important contacts for dimer formation^{11,12}. The binding *in vitro* of repressor to a single operator site has a sigmoidal dependence on repressor concentration, reflecting the requirement for dimer formation^{10,13}.

Interactions between adjacent repressor dimers. Repressor dimers do not bind independently to the three sites in O_R ; rather

	Repressor	<i>cro</i>
Lysogeny	On	Off
Lytic growth	Off	On

Fig. 1 Alternate physiological states of λ . In a lysogen, repressor is made but *cro* is not. During the early stages of lytic growth, *cro* protein but not repressor, is synthesized.

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they bind with positive cooperativity, and this interaction is crucial to repressor action⁴. We define the intrinsic affinity of each site for a repressor dimer as that observed in *in vitro* binding assays using DNA with a single functional site. Such experiments show that the independent affinities of O_R2 and O_R3 are about equal, and ~15-fold less than that of O_R1 . However, when DNA containing all three operators, arranged as on the wild-type genome, is used, repressor binds to O_R1 and O_R2 cooperatively, but O_R3 only binds repressor at much higher concentrations. The explanation for this lies in the interaction of repressors bound at O_R1 and O_R2 : repressor bound to O_R1 raises the repressor-binding affinity of site O_R2 , but not that of O_R3 . The concentration of repressor in a lysogen is such that O_R1 and O_R2 are virtually saturated, but O_R3 is largely unbound^{5,6}.

Interaction between repressor dimers bound to sites O_R2 and O_R3 can be studied when O_R1 is non-functional because of mutation. Repressor cannot then bind to O_R1 , and a repressor bound to O_R2 is free to interact with another at O_R3 . As a result, the affinity of repressor for site O_R3 is raised and that for O_R2 is lowered relative to the wild type. Thus, a repressor dimer bound to O_R2 interacts predominantly with another at O_R1 (on a wild-type template) or with another at O_R3 (on a mutant template in which O_R1 has been destroyed). (We have called this phenomenon 'alternate pairwise cooperativity', and will return to its possible physiological significance later.) The O_R1 - O_R2 interaction predominates on a wild-type operator because O_R1 is the tightest binding site.

We believe that these cooperative interactions are mediated by contacts between the carboxy-terminal domains or the domain connector regions of adjacently bound repressor dimers, because isolated amino-terminal domains bind to the three O_R sites specifically but non-cooperatively⁴. This result argues that the cooperativity is not due to a change in DNA structure induced by repressor binding. In addition, repressor at high concentrations in solution forms tetramers and higher oligomers^{8,10,14}, perhaps using the same contacts as those involved in the cooperative binding to adjacent operator sites. We have suggested^{1,4} that the inability of a repressor dimer bound to O_R2 to interact simultaneously with others at both O_R1 and O_R3 may be due to the limited flexibility and/or size of the connectors.

Activation of P_{RM} . We believe that repressor stimulates transcription from P_{RM} by providing a favourable protein-protein contact with RNA polymerase^{1,6,7}. The evidence for this view includes the following observations: (1) a repressor dimer bound to O_R2 stimulates transcription of P_{RM} ~10-fold both *in vivo* and *in vitro*^{2,6,7}. The primary role of O_R1 in this regard is to ensure that a repressor is bound to O_R2 . (2) Repressor and RNA polymerase bind to DNA cooperatively. For example, using DNase I as a probe, we found that formation of active ('open') complexes of RNA polymerase with P_{RM} is enhanced if repressor is first bound to O_R1 and O_R2 . Conversely, when polymerase

was bound to P_{RM} (in the absence of repressor), the subsequent binding of repressor to O_R2 was similarly enhanced (A.J. and M.P., unpublished results). (In this experiment, O_R1 , P_R and O_R3 were made nonfunctional by mutation.) D. K. Hawley and W. R. McClure (personal communication) have reached similar conclusions and have further found that repressor stimulates formation of 'open' (but not 'closed') complexes of RNA polymerase with P_{RM} (see refs 15-17). (3) Repressor bound at O_R2 is very close to (and may contact) polymerase at P_{RM} . Figure 3b, which shows phosphates in close contact with repressor at O_R2 (and at O_R1) and polymerase at P_{RM} , suggests that the proteins closely approach the same phosphate (and presumably, each other) between bases at positions -36 and -37 from the start-point of P_{RM} transcription (see Fig. 3 legend).

We believe that, as indicated in Fig. 3a, it is the amino-terminal domain of repressor that contacts RNA polymerase at P_{RM} and thus stimulates transcription from this promoter. Four lines of evidence suggest this. First, the purified amino-terminal domain of repressor activates transcription of P_{RM} *in vitro*¹². Second, RNA polymerase bound at P_{RM} enhances the binding of this amino-terminal domain to O_R2 (A.J. and M.P., unpublished results). Third, a repressor fragment comprising the amino-terminal domain of repressor, when produced in large amounts, activates P_{RM} *in vivo*¹². Fourth, a mutant of repressor has been isolated that represses P_R but fails to stimulate P_{RM} (as measured *in vivo* or *in vitro*⁶⁹). The mutant repressor binds to O_R1 and O_R2 cooperatively with an affinity indistinguishable from that of wild-type. Plausibly this mutant maintains the structure(s) required for tight cooperative binding to DNA but lacks those necessary to interact with RNA polymerase. The mutation causes the change gly→arg at position 43 in the amino-terminal domain of repressor⁶⁹.

None of the experiments described above definitively excludes the possibility that repressor bound to O_R2 stimulates P_{RM} by changing DNA structure. However, as repressor has little effect on the pitch of the DNA helix¹⁸, we find no reason to invoke an explanation involving changed DNA structure.

Induction and the role of *cro*

When *Escherichia coli* is treated with any of a variety of mutagens/carcinogens (as defined by the Ames test¹⁹), a set of genes known collectively as the 'SOS functions' is turned on (for reviews see refs 20, 21). One such gene encodes the *recA* protein which, when present in the low levels found in uninduced cells, catalyses genetic recombination. The *recA* protein is also a protease and, after an inducing treatment, it is produced at elevated levels and efficiently cleaves the λ repressor monomer at a specific site between the two domains^{22,23}. (*In vitro*, this reaction requires cofactors presumably similar to those produced *in vivo* in response to inducing treatments.) The free amino-terminal repressor fragments produced by the cleavage cannot dimerize efficiently, and therefore cannot bind tightly to the operator^{11,12}. The over-production and activation of *recA* (both probably required for extensive repressor cleavage) are transient phenomena, lasting for ~1 h after a typical inducing treatment (see refs 20, 24).

As the repressor in a lysogen is inactivated by cleavage, P_R is the first λ lytic promoter to be derepressed. This occurs because repressor binds less tightly to the sites in O_R than to those in O_L , the other major λ operator^{25,26}. The first new phage protein made is the product of the *cro* gene. The *cro* protein is a dimer (monomer length 66 amino acids)^{27,28} which, like the λ repressor, recognizes the three operator sites in O_R . DNA-bound *cro* is centred at each site identically to repressor, covering the same face of the helix and apparently contacting many of the same functional groups²⁶. Unlike the λ repressor, however, *cro* binds to the three O_R sites non-cooperatively, and its order of affinity to the three sites is reversed^{3,4}. That is, *cro* binds most tightly to O_R3 , and only at higher concentrations does it occupy O_R2 and O_R1 . Hence, after destruction of the repressor, *cro* is synthesized and first occupies O_R3 , as shown in Fig. 3e. In this configuration, P_{RM} is repressed and P_R is accessible to RNA

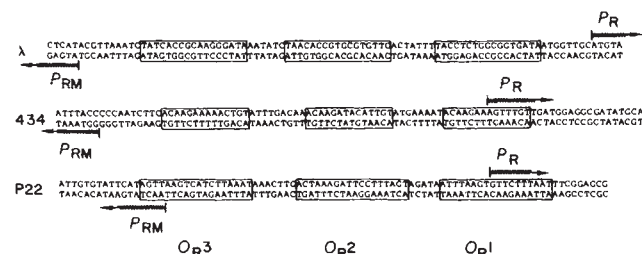


Fig. 2 DNA sequences, operator sites and transcription start-points in the right operators of λ , 434 and P22. O_R1 , O_R2 and O_R3 are the sites recognized by the phage repressors. In the case of λ , we know that the three sites are also recognized by *cro*, and we presume that this is the case for 434 and P22 as well. Arrows mark the transcription start-points for the promoters P_R and P_{RM} . The operator-promoter regions are oriented so that in each case the repressor gene is transcribed to the left and the *cro* gene to the right. The λ operator sites consist of 17 bp; adjacent sites in O_R are separated by 6 and 7 bp. The 434 and P22 operator sites are 14 and 18 bp long, respectively. The 434 spacers consist of 8 and 9 bp, those of P22, 5 and 7 bp. Each operator site exhibits partial twofold rotational symmetry.

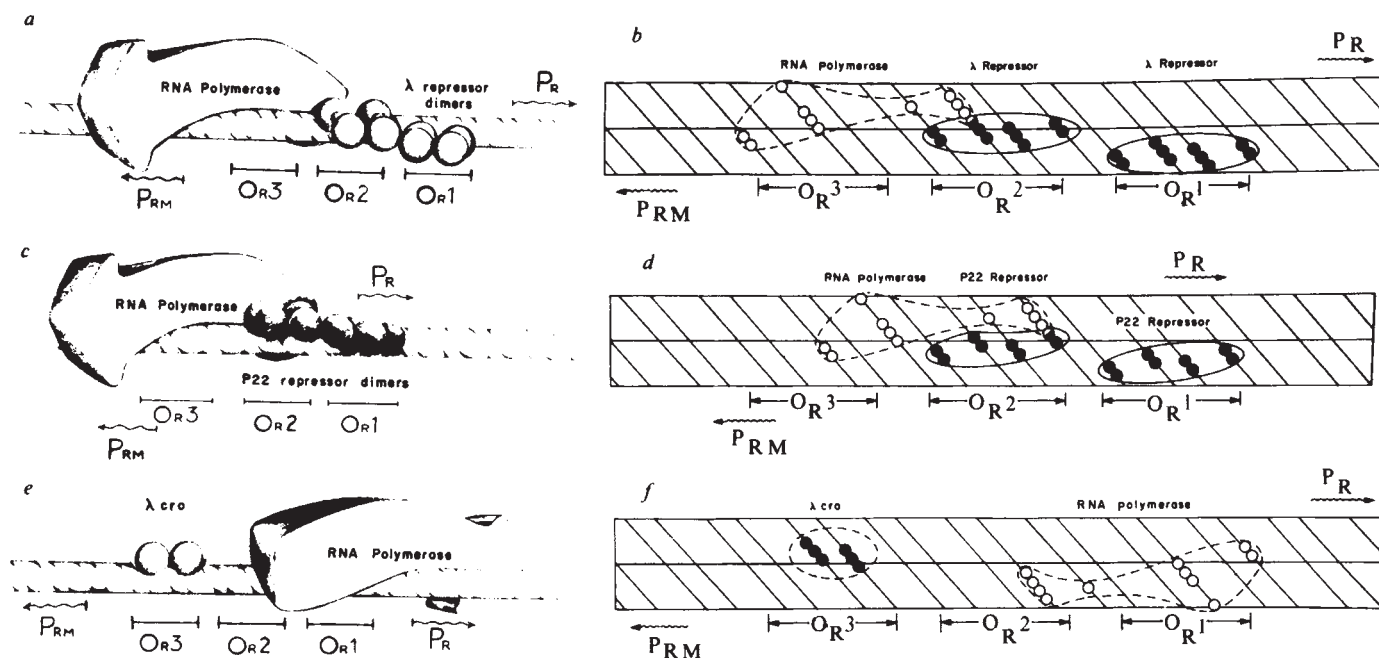


Fig. 3 Configuration of molecules bound to O_R/P_R in a λ lysogen (a, b), in a P22 lysogen (c, d), and during the early stages of λ lytic growth (e, f). a, λ lysogen: dimers of λ repressor are shown bound cooperatively to O_R1 and O_R2 . This arrangement results in repression of P_R and activation of P_{RM} . b, Projection diagram showing the topography of λ repressor dimers bound at O_R1 and O_R2 and RNA polymerase bound to P_{RM} . The λ O_R region of a is shown as a cylindrical projection (adopted from ref. 65) of B-form DNA with 10.4 bp per turn⁶⁶. ●, Phosphates in close contact with bound λ repressor (see below); ○, phosphates believed to be in close contact with RNA polymerase bound at P_{RM} (see below). The solid and broken contour lines were drawn merely to group the phosphate contacts, not to imply the shapes of the DNA interaction sites. Note that the phosphate closely approached by both polymerase and repressor is marked by ●. c, P22 lysogen: dimers of P22 repressors are bound cooperatively to O_R1 and O_R2 , repressing P_R and activating P_{RM} . d, Topography of P22 repressors bound to O_R1 and to O_R2 and RNA polymerase bound to P_{RM} . The symbols are as in b. Note that the relative positioning of P22 repressor at O_R2 and polymerase at P_{RM} differs from that in the case of λ (b). e, λ lytic growth: after induction of a λ lysogen, *cro* proteins binds to O_R3 thereby repressing P_{RM} and permitting P_R transcription. The precise positioning of these regulatory molecules on the DNA helix is based on the results of chemical probe experiments, some of which are summarized in b, d and f. f, Topography of λ *cro* bound to O_R3 and RNA polymerase bound to P_R . Symbols are as above. *cro* makes four fewer phosphate contacts at each operator site than does repressor. The phosphates in close contact with a bound λ repressor²⁶, λ *cro*²⁶ and P22 repressor⁴² were determined using the method of Maxam and Gilbert (personal communication) and Siebenlist and Gilbert⁶⁷. The locations of phosphates in close contact with RNA polymerase were inferred from an analogy with the results of chemical probe experiments performed on two other *E. coli* promoters⁶⁵, *lacUV5* and T7A3. Because *E. coli* promoters share base-pair homology, we were able to infer the likely contact points for P_R and P_{RM} of λ and P22 as summarized here.

polymerase^{3,6}. (When bound to O_R3 , *cro* has no effect on P_R which, unlike P_{RM} , requires no auxiliary factor to function maximally.) Soon after lytic growth has commenced, as the concentration of *cro* increases, it turns down transcription from P_R by binding to O_R1 and O_R2 . This repression of early functions (including the production of *cro*) is essential for lytic growth²⁹.

Two additional experiments (A.J., R. Maurer and M.P., unpublished results) emphasize the importance of the *cro*- O_R3 interaction for induction. In the first, we studied λ lysogens bearing a plasmid which directs synthesis of *cro* under the control of the *lac* promoter. Synthesis of *cro* from the plasmid causes the lysogen to induce, even in the absence of repressor inactivation. This effect requires that the prophage carries an intact O_R3 . We imagine that in this experiment, *cro* binds to O_R3 and shuts off P_{RM} , and thus the production of repressor. Then, as the cells divide, repressor is diluted and induction occurs. The second experiment shows that binding of *cro* to O_R3 is critical for efficient induction. We found that prophages mutated at O_R3 in such a way that *cro* binds poorly, induce much less efficiently in response to UV irradiation than do wild-type prophages.

Once a phage genome reaches the state shown in Fig. 3e, we believe that it is committed to grow lytically. Hence, we view the two configurations of Fig. 3a and e as alternative states of the phage. Following induction, a prophage can be 'frozen' in the otherwise transient lytic state shown in Fig. 3e if it carries mutations which prevent expression of lytic genes other than *cro*³⁰. When the lysogenic bacterium bearing the mutant prophage is induced, it switches from the normal state where repressor but not *cro* is synthesized, to a new state in which *cro* but not repressor is synthesized (Fig. 3e). This new state, called 'anti-immune', like the normal lysogenic state, is inherited by subsequent generations.

Related phages

Which of the attributes of λ described above are vital to its life-cycle, and which are peculiar, perhaps accidental, to the λ case? To explore these questions, we now compare λ with two other phages, 434 and P22, to see which features are held in common. These other phages share with λ many basic biological properties; in particular, they all form UV-inducible lysogens. The genomes of the coliphages 434 and λ and of the *Salmonella* phage P22 are similarly organized, and share regions of homology^{31,32}. Despite these similarities, the nucleotide sequences of the operators (Fig. 2) and the amino acid sequences of the repressors and *cro* proteins differ substantially. In particular, the repressor and *cro* of one phage do not recognize the operators of the others.

Nevertheless, we now argue that despite differences in operators, promoters and regulatory proteins, the following description holds for all three phages. The right-hand operator contains three repressor binding sites, two of which, O_R1 and O_R2 , are filled by repressor in a lysogen. This state depends on cooperative interactions between adjacent repressor dimers. Repressor monomers have two structural domains and are in equilibrium with dimers, the DNA-binding form. Repressor dimers occupying sites O_R1 and O_R2 turn off transcription from P_R and turn on that from P_{RM} . On UV induction, the repressor is cleaved and thereby inactivated, and the first protein synthesized from P_R is *cro*. The *cro* protein binds first to O_R3 to turn off repressor synthesis and later binds to O_R1 and O_R2 to decrease early lytic transcription.

The pictures we have developed for 434 and P22 are based on extensive experiments, *in vivo* and *in vitro*, similar to those performed for the analysis of λ (see ref. 1). In each case, plasmids that direct synthesis of large and variable amounts of the regulatory proteins were constructed by recombination *in*

vitro. The effects of repressor *in vivo* were analysed using these plasmids and other DNA molecules in which transcription of the *lacZ* gene was directed by a phage P_{RM} or P_R . The response of the wild-type and various mutant derivative promoters to different amounts of repressor was analysed. The interaction of the repressors, of *cro* proteins and of RNA polymerase with wild-type and mutant operators, as well as other properties of these proteins, were studied *in vitro* using purified components. By combining results from various such experiments, a coherent picture has emerged in each case.

Similarities in repressor structures. Like the λ repressor, the P22 and 434 repressors are folded into two structural domains which can be separated by proteases, including activated *recA* (refs 11, 33) and J. Anderson and M.P.; J. De Anda and R.S.; and R. Yocum and M.P., unpublished results). The amino-terminal fragments of 434 and P22 repressors bind specifically to their operators (J. Anderson and M.P.; J. De Anda, A.P. and R.S., unpublished results). Although the evidence is inconclusive, it seems that the P22 and 434 repressors must dimerize before binding to their operators^{9,34}.

The λ , 434 and P22 repressors share extensive homology in their carboxy-terminal regions (refs 35, 36; R. Yocum and M.P., unpublished results). When the sequences are aligned, ~30% of the amino acids are identical. The amino-terminal domains of the three phage repressors (and of the *cro* protein—see below) show considerably less, but still significant, homology (R.S., R. Yocum, A.P. and R. Doolittle, unpublished results). Of particular interest is a conserved Ala-Gly near the middle of each repressor. The *recA* protease cleaves this bond in all three repressors (ref. 33 and R. Yocum and M.P., unpublished results). We surmise that these homologies reflect, at least in part, structures that are recognized and cleaved by the *recA* protease to effect phage induction (see ref. 33). We note that a cellular repressor of UV-inducible genes, the product of the *lexA* gene, is also cleaved by the *recA* protein; it shares substantial carboxy-terminal homology with the phage repressors and carries an Ala-Gly in or near the *recA* cleavage site (refs 37, 38 and R. Yocum, R. Brent and M.P., unpublished results).

Similarities in operator structure and repressor binding. Although the λ , 434 and P22 O_R s differ in DNA sequence, they are arranged in a strikingly similar way: each consists of three homologous, partially symmetric repressor binding sites (Fig. 2 and refs 1, 39–41). As in the case of λ , repressor dimers of 434 and P22 do not bind independently to their three operator sites; rather they fill sites O_{R1} and O_{R2} cooperatively and coordinately^{41,42}. In P22, this cooperativity presumably results from protein-protein interactions between carboxy-terminal sequences of adjacently bound repressor dimers, because as with λ , the isolated amino-terminal domains bind specifically but non-cooperatively to the O_R of P22 (J. De Anda, A.P. and R.S., unpublished results). As for λ , so with 434 and P22: if repressor cannot bind to O_{R1} because that site is mutant, the repressors fill sites O_{R2} and O_{R3} cooperatively^{41,42}.

The intrinsic affinity of each phage repressor for its operator sites, together with the cooperative interactions, ensure that for all three phages, repressor binds with the affinity order $O_{R1} \approx O_{R2} > O_{R3}$. In lysogens of λ , 434 or P22, the repressor level is such that almost all templates bear repressors at O_{R1} and O_{R2} and that O_{R3} is free of repressor in a significant fraction of cells^{5,6,41,42}.

Common patterns of gene control by repressor. Repressors bound to O_{R1} and O_{R2} in both P22 and 434 elicit the same transcriptional response as does λ repressor bound to the corresponding sites. That is, in each case, repressor turns off P_R and turns on P_{RM} (refs 6, 41, 42). However, this similarity is deceptive and, indeed puzzling, because the overlaps between the promoters and operators are different in the three cases. For example, O_{R2} in P22 is 11 base pairs (bp) closer to the transcriptional start of P_{RM} than is the O_{R2} of λ to its P_{RM} (Fig. 2). Nevertheless, in P22 as in λ (as well as in 434), repressor bound only to O_{R2} activates P_{RM} . At first glance, this is surprising because, as we have previously described for λ , very small

differences in the position of repressor can qualitatively change the effect on transcription. For example, λ repressor bound to O_{R2} alone (a condition achieved with appropriate operator mutants) turns off P_R and turns on P_{RM} , even though it is positioned exactly 1 bp closer to the former than the latter. We have argued that this 1-bp difference enables repressor to contact polymerase and aid its binding to one promoter (P_{RM}), excluding its binding to the other (P_R). Nevertheless we believe that in spite of differences in promoter-operator overlap, a common mechanism of positive (as well as of negative) control obtains for λ and P22.

Positive control: In P22 as in λ , a protein-protein contact could be made between the amino-terminal domain of a repressor bound at O_{R2} and an RNA polymerase molecule bound at P_{RM} (see Fig. 3a, c). Moreover, the same region of RNA polymerase (shaded in Fig. 3) plausibly could be contacted in the two cases. This hypothesized site of contact is near a phosphate group which, according to the chemical probe experiments, is closely approached by both repressor at O_{R2} and polymerase at P_{RM} (compare Fig. 3b, d). In any case, the spatial relationship of polymerase and repressor differs in the two cases: for P22, repressor (at O_{R2}) and RNA polymerase (at P_{RM}) cover different faces of the same two turns of the DNA helix, whereas in λ the molecules are arranged more nearly in tandem. In the case of 434, the distance between O_{R2} and P_{RM} is very close to that for λ , and it is likely that the relationship of repressor at O_{R2} and polymerase at P_{RM} is the same for λ and 434 (see Fig. 2). **Negative control:** Repressor bound to O_{R1} and O_{R2} excludes binding of RNA polymerase to P_R , a point readily visualized for the λ case by comparing Fig. 3b and f. 434 and P22 repressors also exclude binding of RNA polymerase to P_R , but in these cases, different sets of DNA contact points are masked^{41,42}. As expected from this model nuclease probe experiments (performed with λ and P22) show that binding of RNA polymerase to P_R and repressor to O_{R1} and O_{R2} are mutually exclusive^{26,42}. Moreover, in the case of λ , it has been explicitly shown that repressor bound either to O_{R1} or to O_{R2} represses P_R ⁶.

Common mechanism of *cro* action. Like λ , 434 and P22 also encode *cro* proteins. The 434 *cro* protein, roughly the same size as that of λ , has been isolated and partially characterized⁴³. It binds specifically to (or near) the 434 operator. Strains bearing mutated 434 and P22 prophage can exist stably in the 'anti-immune' state (see above) in which repressor synthesis is turned off, but *cro* synthesis persists^{30,44}. This suggests, by analogy with λ , that the *cro* proteins from these phages bind to O_{R3} more tightly than to the other two sites in O_R , and that *cro* bound to O_{R3} would repress P_{RM} but allow transcription of P_R .

An intriguing fact has recently emerged from a comparison of the λ , 434 and P22 *cro* and repressor amino acid sequences (R.S., R. Yocum, A.P. and R. Doolittle, unpublished results). When the *cro* sequences are aligned with those of the repressor amino-terminal domains, significant homologies are revealed. Some positions are conserved in all six proteins; others are occupied by amino acids of a similar chemical type. The homology between individual *cro* and repressor sequences is in some cases very strong, and in others, much weaker. For example, repressor and *cro* from 434 are strikingly similar in sequence (ref. 45 and R. Yocum, unpublished results) whereas λ repressor and λ *cro* show little obvious homology to one another, a point noted previously by us and others^{1,28,35}. In particular, the 10-amino acid α helix of λ *cro* proposed by Anderson *et al.*⁴⁶ to fit into the major groove of operator DNA shows little homology with the corresponding region of λ repressor. However (as noted above), these regions (identified by homology) are very similar in *cro* and repressor of 434. It remains to be seen how these similarities and differences in amino-acid sequence contribute to the different order of binding, and hence physiological effects, of the three *cro*/repressor pairs. In any case, the similarities between the phage repressor and *cro* sequences suggest a common evolutionary origin for the six regulatory proteins.

Cooperativity and phage induction

Here we argue that the features that are common to λ , 434 and P22, including the tripartite operator, the various forms of cooperativity and the action of *cro*, enable the phage to switch from one state (lysogeny) to the another (lytic growth) in response to a transient signal.

Repressor dimerization and cooperative binding of dimers to adjacent sites. Why are two sites, O_{R1} and O_{R2} , used to mediate the effects of repressor in a lysogen? Why does repressor dimerize and why do two dimers fill sites O_{R1} and O_{R2} cooperatively? At first glance, it might seem that a stable dimer binding tightly to a single site (O_{R2}) would suffice. We believe that the dimerization and subsequent cooperative repressor binding to two adjacent sites ensures tight repression but maintains the system in a state poised to respond dramatically to a rather modest decrease in repressor concentration.

This conclusion is illustrated in part by Fig. 4, which indicates that P_R repression is a steep function of repressor concentration. This curve was calculated from data describing the binding of repressor to various wild-type and mutant templates *in vitro* (see Table 1 and Fig. 4 legend). Our measurements and calculations⁴⁷ indicate that in a lysogen, the degree of repression of P_R is at least 99% (see Fig. 4 legend). According to the curve, inactivation of 90% of the repressor would suffice to derepress P_R to ~50% of its maximal level. This response is in striking contrast to that produced by a system involving stably oligomeric repressor binding to a single operator site (as exemplified by *lac*) which maintains an equivalent amount of

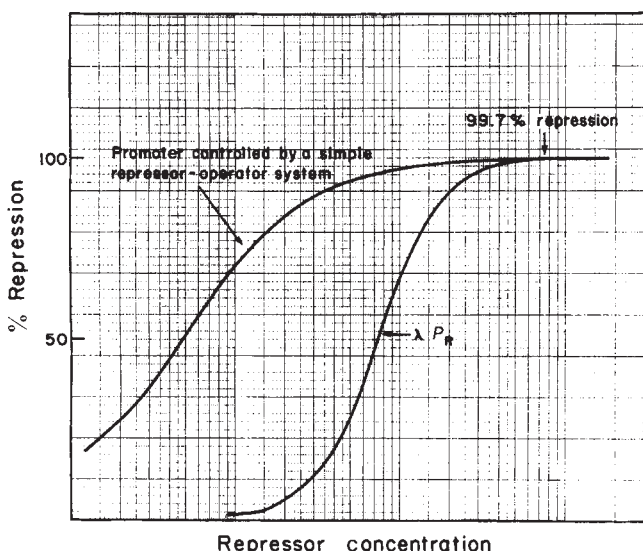


Fig. 4 Theoretical repression curves for λP_R and a simple repressor-operator system such as *lac*. The curves show the extent of repression as a function of total repressor concentration. They were calculated as follows. λP_R : at each total repressor concentration, the concentration of repressor dimers was calculated from the known equilibrium dissociation constant ($K_D = 2 \times 10^{-8} M$)¹⁰. The distribution of repressor dimers among each of the eight possible states of the operator (Table 1) was calculated from a knowledge of the relative energy of each state using standard methods of statistical thermodynamics⁴⁷. The energies of each of the eight states of Table 1 were deduced from DNA binding studies carried out *in vitro*⁴. A repressor dimer bound either to O_{R1} , O_{R2} or to both sites, will repress P_R ⁶. For any repressor dimer concentration, therefore, the fraction of O_R templates with P_R repressed is given as the sum of states 1, 2, 4, 5, 6 and 7 from Table 1. Simple repressor-operator system: for comparison we show a theoretical repression curve for the case of a protein (of a stable oligomeric form) binding to a single DNA site (that is, *lac* repressor binding to its operator). The curve was generated from the simple relation, $OR/OR_T = 1/(1 + K_D/R_T)$ where OR/OR_T is the fraction of operators occupied by a repressor, R_T being the total repressor concentration, and K_D is the dissociation constant describing the reaction $RO = R + O$ (ref. 68). The two curves in the figure were aligned at the 99.7% repression point. We believe this to be a minimum estimate of the extent of repression found in a λ lysogen. This value was derived from the knowledge⁵ that in a lysogen, O_{R3} is ~20% repressed. According to our calculations, when O_{R3} is 20% repressed, P_R is at least 99.7% repressed. We have no direct way of measuring accurately the extent of repression actually found in a lysogen. However, we know that the extent of repression of the *lac* operon *in vivo* is ~99.9%.

Table 1 Eight possible configurations of repressor at O_R

State	Configuration O_{R3} O_{R2} O_{R1}	Free-energy contributions	Total free energy (kcal mol ⁻¹)
0		(reference state)	0
1		ΔG_1	-11.6
2		ΔG_2	-10.1
3	+	ΔG_3	-10.1
4		$\Delta G_1 + \Delta G_2 + \Delta G_{12}$	-23.7
5	+	$\Delta G_1 + \Delta G_3$	-21.7
6	+	$\Delta G_2 + \Delta G_3 + \Delta G_{23}$	-22.2
7	+	$\Delta G_1 + \Delta G_2 + \Delta G_3 + \Delta G_{12}$	-33.8

+ Indicates that the corresponding site is occupied by a repressor dimer. The free energies of each state (relative to the reference state) are listed. ΔG_1 , ΔG_2 and ΔG_3 are the intrinsic interaction energies of repressor dimers binding to each of the three sites. ΔG_{12} is the net interaction energy supplied by the cooperative interaction between a repressor dimer at O_{R1} and one at O_{R2} . ΔG_{23} is similarly defined. These values, measured in 'physiological conditions' (200 mM KCl, 37 °C) are $\Delta G_1 = -11.6$ kcal mol⁻¹, $\Delta G_2 = \Delta G_3 = -10.1$ kcal mol⁻¹, $\Delta G_{12} = \Delta G_{23} = -2.0$ kcal mol⁻¹ (ref. 4) (see Fig. 4 legend).

repression (see Fig. 4). In this case, 50% induction requires inactivation of a much larger fraction of repressor (>99%), and inactivation of 90% of the repressor would result in only ~3% derepression and thus induction. There is evidence that inactivation of 90% of the λ repressor in a lysogen produces highly efficient induction²⁴. Both repressor dimerization and cooperative binding to O_{R1} and O_{R2} contribute to the steep slope of Fig. 4 (ref. 47).

P_{RM} activation and the action of *cro* protein. Once P_R of a prophage is derepressed, why does the phage not simply re-establish repression as the inducing signal decays? We believe that the dependence of P_{RM} activity on bound repressor and the action of *cro* prevent the re-establishment of lysogeny and promote lytic growth. As repressor present in a lysogen is inactivated, it vacates O_R . A template lacking a repressor at O_{R2} no longer directs synthesis of repressor efficiently, and thus the rate of repressor synthesis declines. At the same time, *cro* is synthesized, and by binding first to O_{R3} , turns off repressor synthesis and promotes lytic growth.

Thus in summary the three related phages, λ , 434 and P22, encode specific repressor and *cro* proteins that interact in each case with a key regulatory sequence, termed the right operator. We find that despite detailed differences in mechanism, in each case repressor in a lysogen binds cooperatively to the tripartite operator, turning off lytic genes and stimulating transcription of its own gene. We have argued that a common molecular mechanism of positive and negative control obtains in each case, despite differences in operator-promoter overlap. Moreover, we argue that the common features of repressor action, including in each case its cooperative interaction with a tripartite operator, have been maintained to ensure the switch from lysogenic to lytic growth. Thus, in a lysogen, repressor is bound predominantly to O_{R1} and to O_{R2} , thereby repressing P_R and activating P_{RM} ; to a lesser extent repressor is bound to O_{R3} , thereby turning down repressor synthesis.

The lysogenic state is stably inherited; lysogens spontaneously induce only about once per 10^5 generations. On exposure of lysogens to inducer, cleavage of repressor commences. A 5–10-fold drop in repressor level triggers a dramatic change of state as O_{R1} and O_{R2} are vacated: because repressor synthesis depends on autogenous stimulation, its rate of synthesis decays rapidly. Moreover, induction of P_R results in synthesis of *cro*, a protein required for lytic growth, which binds to O_{R3} , turns off P_{RM} , and thereby completes the switch to lytic growth. The various forms of protein-protein interaction involving repressor ensure stability of the lysogen but permit P_R to begin functioning in response to a moderate decrease in repressor level.

Further considerations

Role and positioning of O_{R3} . We have explained that the two sites O_{R1} and O_{R2} and their interaction serve an important

biological function that could not be met by a single repressor binding site. We have also described a role for O_{R3} , that is, turning off repressor synthesis by *cro* and thus promoting lytic growth. We now suggest two additional functions for O_{R3} .

First, repressor can also bind to O_{R3} and turn off transcription of its own gene. We have found that in lysogens, O_{R3} of λ is ~20% occupied (and O_{R3} of P22 slightly more), whereas sites O_{R1} and O_{R2} in both λ and P22 lysogens are almost completely filled^{5,6,41,42}. The negative autogenous control mediated by repressor binding to O_{R3} should help to maintain constant repressor concentrations, but apparently the effect is not dramatic. For example, we have found that a fivefold increase in λ repressor concentration decreases the rate of repressor synthesis only by ~20% (ref. 5); we do not know whether this response is physiologically important. Perhaps slight decreases in repressor levels that otherwise might trigger induction are compensated for by increased repressor synthesis as O_{R3} is vacated. It is possible that negative autogenous control in 434 is more extensive than in the other two phages (see ref. 9), but our understanding of the situation is incomplete.

Second, as we have mentioned elsewhere¹, we believe that O_{R3} helps to prevent the accumulation of λ virulent mutants—variants that grow lytically (and only lytically) on lysogenic as well as on non-lysogenic cells, and thereby destroy the lysogenic system. A requirement for virulence is that transcription from P_R proceed efficiently in the presence of repressor at the concentration found in a lysogen. In general⁴⁸, two sites in O_R must be mutant, either one in O_{R1} and another in O_{R2} or one in O_{R1} and another in O_{R3} .

We understand this requirement for two mutations to be as follows. (1) If a phage acquires a mutation in O_{R1} alone, P_R will nevertheless be repressed because the cooperative interaction between repressor dimers at O_{R2} and O_{R3} ensures that O_{R2} will be occupied. Addition of a second mutation in O_{R2} or in O_{R3} is necessary for P_R to escape repression. This hypothesized role of O_{R3} (but not the first two described above) requires that O_{R3} be positioned close to O_{R2} so that the necessary cooperative interaction can occur. (2) If a phage acquires a mutation in O_{R2} , the high intrinsic affinity of O_{R1} for repressor nevertheless ensures that P_R will be repressed, and an additional mutation in O_{R1} is required. Thus, the pattern of intrinsic affinities and alternate cooperative repressor binding to the three sites imposes the requirement that two mutations, rather than one, must be accumulated for O_R to escape repression. Furthermore, phage that have acquired one operator mutation (in either O_{R1} or O_{R2}) cannot lysogenize (because occupancy of O_{R1} and O_{R2} , but not of O_{R3} , is required for lysogeny) and such phage are thereby presumably at a selective disadvantage compared with wild-type.

Efficiencies of λ and *lac* repressor. The affinity (K_D) of a λ repressor dimer for O_{R1} on a wild-type template in 'physiological conditions' (37 °C, 0.2 M KCl) is 3×10^{-9} M (refs 4, 10, 13). The other, well-studied repressor, the *lac* repressor, binds to its operator much more tightly in these conditions ($K_D = 10^{-12}$ M)⁴⁹. Nevertheless, as argued in the text, we believe that λ repressor in a lysogen turns off P_R about as efficiently as *lac* repressor turns off the *lac* promoter (repression $\geq 99.7\%$).

The λ repressor achieves a similar degree of repression despite a lower binding constant because of the following factors. First, the λ repressor is present at 5–10-fold higher concentration in a cell than is *lac* repressor. (There are ~200 monomers of λ repressor (10^{-7} M) in a lysogen^{8,9,24,50} and the K_D describing dimer formation is 2×10^{-8} M (ref. 10), whereas the concentration of *lac* repressor stable species (tetramer) is 10–20 molecules per cell^{51,52}.) Second, the affinity of *lac* repressor for non-operator DNA may decrease its effective concentration in *E. coli* relative to λ repressor. For *lac* repressor, the ratio of affinities for nonspecific to operator DNA is $\sim 10^{-8}$ (ref. 49). The binding constant of *lac* repressor for random 20-bp non-operator sequences (10^{-4} M), taken with the

total concentration of such sequences in *E. coli* (10^{-2} M), suggests that an appreciable fraction of repressor is sequestered on nonspecific DNA^{49,53}. Linn and Riggs⁴⁹ have argued that this effectively weakens the binding constant of *lac* repressor by two orders of magnitude. We know that for λ repressor, the ratio of nonspecific to specific DNA binding is $\sim 10^{-8}$ M at low salt and low temperature¹⁰. Assuming that this ratio is the same in 'physiological conditions' (see above), we estimate that little or none of the λ repressor in a lysogen is bound to non-operator DNA. Third, a consequence of repressor dimerization and of cooperative binding of repressors to O_{R1} and O_{R2} (see Fig. 4) is that 99.7% repression is reached when the concentration of repressor is ~12-fold above that needed to fill half the sites (that is, the apparent K_D), and this is the amount of repressor found in a lysogen. In the *lac* case, this degree of repression is achieved only when the concentration of repressor exceeds the K_D 300-fold.

Positive control. We have described a particular mechanism whereby the phage repressors mediate positive control of transcription in λ . Note that we have paid little attention to an alternative mechanism, which we now discuss.

It is conceivable that RNA polymerase bound to the strong promoter, P_R , would exclude binding of polymerase to the weak promoter, P_{RM} , and that the role of repressor in positive control is to prevent binding of polymerase to P_R .

This model deserves serious consideration for λ , 434 and P22 because in each case the two promoters, P_R and P_{RM} , are adjacent. A series of experiments argues strongly that in λ , this model is excluded. Polymerase bound at λP_R has little inhibitory effect on binding of polymerase to P_{RM} , and removing P_R does not activate P_{RM} ⁷. In the case of P22 and 434, P_R and P_{RM} are significantly closer than in λ , and although we have no definitive evidence, we believe it likely that in each case polymerase bound to one inhibits binding of polymerase to the other^{41,42}. Nevertheless, deletion of P_R of P22 does not activate its P_{RM} : rather, as in λ , repressor must be bound to the DNA for P_{RM} stimulation. Thus, in λ , the repressor provides an essential contact with polymerase at P_{RM} , whereas it is likely that in P22 and 434, the repressor both excludes a competing polymerase and provides the necessary protein contact with polymerase at P_{RM} .

Analogy with haemoglobin. A haemoglobin tetramer bearing a bound oxygen has a higher affinity for oxygen than does a haemoglobin tetramer bearing no oxygen⁵⁴. As a result, a small change in oxygen pressure can cause a dramatic change in oxygen binding to haemoglobin. The interaction of the λ operators with λ repressor and of haemoglobin with oxygen are formally analogous. As shown in Table 1, the energy of interaction of repressor for O_{R1} alone is -11.6 kcal mol⁻¹, and that for O_{R2} alone is -10.1 kcal mol⁻¹. The net interaction energy between adjacent repressor molecules is -2 kcal mol⁻¹, which means that the effective energy of binding to the second site on a wild-type template is -12.1 kcal mol⁻¹. Thus, an operator containing a bound repressor has a higher affinity for a second repressor than does an operator bearing no repressor. As a consequence, small changes in repressor concentration can have a dramatic effect on repressor binding to the operator (see Fig. 4). An important difference in the analyses of λ and haemoglobin is that in the latter case, one must measure the total amount of ligand (oxygen) bound. In contrast, for λ , we can quantitate binding of the ligand (repressor) to each of the three sites in the operator. This greatly simplifies analysis of mechanism (see ref. 47).

Other cases. Interactions between adjacent DNA-bound proteins probably also play an important part in both prokaryotic (see, for example, refs 70–73) and in eukaryotic gene regulation. For example, transcription of the SV40 early genes (including that which encodes T antigen) is repressed by the T antigen^{55–59}. This protein binds to three sites near the early transcription start-point. Binding of the purified protein to the middle site is enhanced by previous binding to the first⁶⁰. There is evidence⁶⁰ that this cooperative binding is mediated by pro-

tein-protein interactions between adjacent T-antigen molecules.

In another case, transcription of the 5S gene of *Xenopus* in partially fractionated oocyte extracts is stimulated by a protein that binds specifically to a site within the gene⁶¹. One interpretation of the experiments of Sakonju *et al.*^{61,62} is that the function of the stimulatory factor is to promote binding of RNA polymerase to the (relatively nonspecific) adjacent DNA sequence.

In prokaryotes, as well as eukaryotes, transient signals can induce epigenetic changes which are then stably inherited^{63,64}. The phage cases we have analysed demonstrate one molecular mechanism for ensuring such a response. More generally, it is

plausible that at one or more stages during the development of higher organisms, differences between daughter cells depend on the asymmetric distribution of cytoplasmic determinants formerly present in the parental cell. Our analysis of the λ case shows how a relatively small (<10-fold) change in such factors can cause a drastic epigenetic change.

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ARTICLES

Oscillatory zoning: a pathological case of crystal growth

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A new theory of oscillatory zoning in naturally grown plagioclase crystals is presented. This describes explicitly the coupling between the interface kinetics and the diffusion of chemical species in the melt. The crystal growth rate R responds with a finite delay time to concentration changes at the interface. Thus the growth rate cannot be simply some function of the supersaturation. Oscillatory zoning occurs when growth rate is low. This theory accounts for the absence or extreme rareness of oscillatory zoned plagioclase crystals in laboratory growth experiments.

PLAGIOCLASES are usually compositionally, and thus optically, zoned. On large scale irregular and abrupt changes in composition (stepped zoning) are often superimposed almost

periodic variations (oscillatory zoning). This 'mineral stratigraphy' has long interested petrologists because it offers a potentially useful record of environment changes during

crystallization¹⁻⁷. It is generally accepted that stepped zoning is created by physicochemical variations in the magma whereas oscillatory zoning is a local phenomenon due to crystal growth processes. However, no quantitative analytical theory has been developed, and the available theories do not explain why the plagioclase-melt system is one of the few natural systems to exhibit this phenomenon.

Zoning patterns in plagioclase crystals have been described elsewhere^{1-4,8-12}. Typical oscillatory zoning characteristics are illustrated in Fig. 1: oscillations occur as groups separated by abrupt changes. The sequence from inner to outer groups generally displays a trend towards greater albite content. The oscillation width is almost constant for a given group but may vary from group to group in the 10–30 μm range. Each concentration peak has a characteristic shape, usually a smooth Ab increasing flank followed by a steep Ab decreasing flank, although there may be no asymmetry in some cases⁷.

We do not discuss the arguments based on constitutional supersaturation which favour an 'internal' origin for oscillatory zoning (see, for example, refs 4, 6 and 7) but from an observational point of view, it is remarkable that no inter-crystal correlation of oscillation bands has been reported¹³. Several authors^{6,14,15} have demonstrated the existence of concentration gradients before growing plagioclase crystals and stressed the

fundamental role of chemical diffusion. There is clear evidence for a near-crystal layer enriched in elements with solid-liquid partition coefficients smaller than 1 (Mg, Fe, Si, Na)⁶.

The phenomena which occur during crystallization from a liquid can be broadly classified as interactions between three important variables: (1) the crystallographic features of the growing crystal and the associated effects on the interface kinetics, (2) the temperature distribution in the liquid, (3) the distribution of elements in the liquid.

In the present context, heat diffusion is much more efficient than chemical diffusion and temperature is not a limiting variable for the low growth rates considered⁶. The competitive character of interface kinetics and chemical diffusion alone does not explain the phenomenon of oscillatory zoning⁶. Consider a newly nucleated crystal: there is no diffusion layer in front of the interface, and growth begins with a fast increasing Ab concentration. Eventually, a steady-state regime should be reached with equal growth and diffusion rates. Kinetic effects may explain why this may not occur.

Equations for plagioclase growth

We now consider the equations which govern the interface kinetics-chemical diffusion interaction. Assuming a flat interface, we consider a coordinate system which moves with the crystal surface, and restrict the discussion to one-dimension, two-component fickian diffusion for simplicity. Strictly, our single independent component is that eigen-vector of the diffusion coefficient matrix which is associated with its lowest eigenvalue D , but as it is comparatively Si-rich and Al-poor, it will be labelled 'albite' for clarity. Albite concentration in the melt at a distance x from the interface, $C(x, t)$, is governed by:

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} + R \frac{\partial C}{\partial x} \quad (1)$$

where $R(t)$ is the crystal growth rate. If slower solid diffusion is neglected, mass balance at the interface reads:

$$R(C_0 - C_s) = -D \left. \frac{\partial C}{\partial x} \right|_{x=0} \quad (2)$$

where $C_0(t) = C(0, t)$ and $C_s(t)$ stand for liquid and solid Ab concentrations at the interface. The second boundary condition is a 'far away fixed concentration' condition:

$$C(x_1, t) = C_m \quad (3)$$

where x_1 is the distance at which mean magma Ab concentration C_m is maintained. For simplicity, x_1 is taken as infinite throughout.

For given initial conditions, two more boundary conditions must be specified. In the limiting case of instantaneous interface kinetics (when growth is controlled by diffusion in the vicinity of the interface), these are the thermostatic equations $C_0 = C_L$ and $C_s = C_S$, where C_L and C_S are the liquidus and solidus Ab concentrations. In the other limiting case of instantaneous diffusion (interface kinetics controlled growth), equations (1) and (2) degenerate and C_s and R are functions of $C_0 = C_m$. In the more intricate intermediate situations, the two required conditions must link C_s and R to C_0 and t , but there are few constraints from experiments or theory (see refs 16 and 17 for discussions). There is, however, some experimental evidence that the partition coefficient $K = C_s/C_0$ remains approximately constant even when R changes by an order of magnitude¹⁸.

Most theories explicitly or implicitly state that C_s and R are single-valued functions of C_0 , as they are in the limiting case of instantaneous diffusion (well-stirred solutions or melts). But, complicated though these functions may be, equations (1) (2) and (3) cannot produce oscillations, because of the presence of only a first-order time derivative (this is at variance with mechanical or electrical oscillators for which a second-order or two coupled first-order differential equations are involved).

Bottinga *et al.*⁶ and Sibley *et al.*⁷ suggest a hysteresis behaviour for R . Oscillatory zoning requires a jump from very slow growth

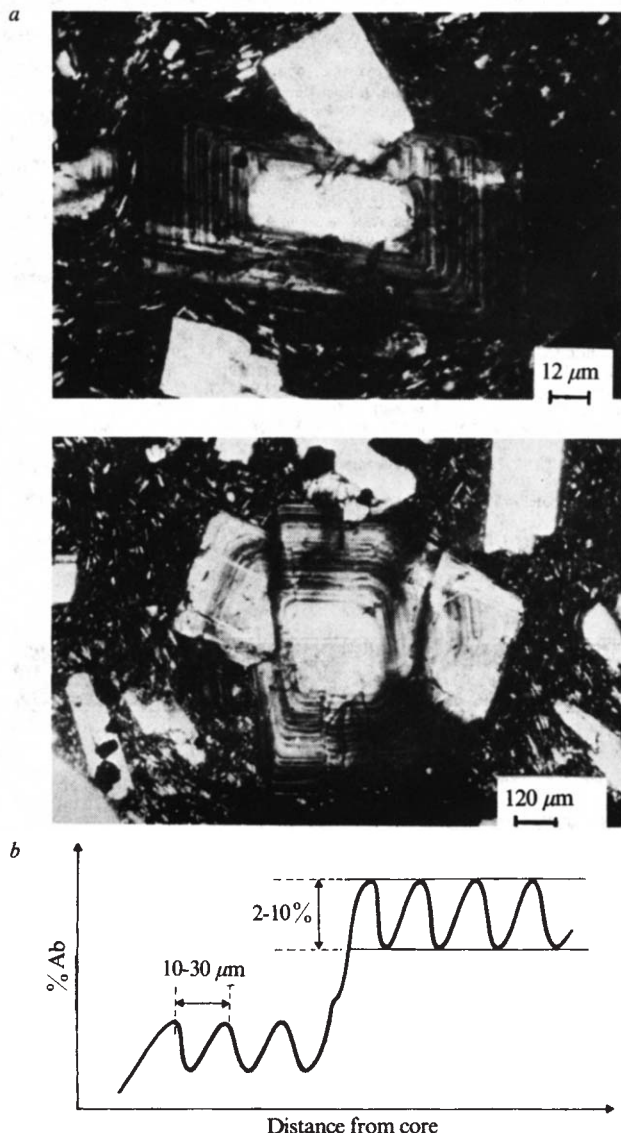


Fig. 1 *a*, Two examples of oscillatory zoning in plagioclase crystals. Samples are basaltic andesites from the Colima volcano (Mexico) (photographs provided by C. Bollinger). *b*, Cross-section of an oscillatory zoned portion of a plagioclase crystal: schematic plot of composition versus distance (from refs 6, 7).

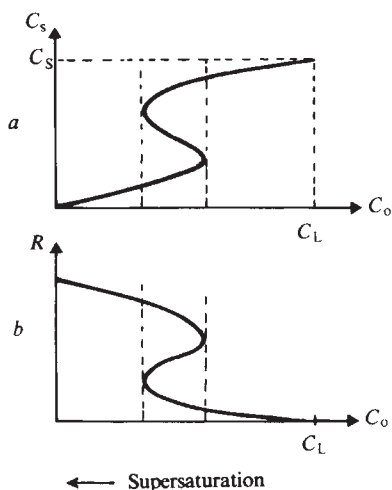


Fig. 2 According to Haase *et al.*²¹, the albite content C_s (a) of a growing plagioclase crystal and its growth rate R (b) may be triple-valued functions of C_0 (the albite content at interface) over a finite interval of supersaturations. This figure is not drawn to scale.

to no growth, which in turn has to wait for a new nucleation step. But their discussions offer no clear explanation for such a jump and rely on an 'interface diffuseness' concept¹⁹ which seems questionable²⁰.

The kinetic theory proposed by Haase *et al.*²¹ considers C_s and R as functions of C_0 and C_s , then predicts that C_s and thus R are triple-valued functions of C_0 on a certain interval of C_0 values (Fig. 2). They argue that C_s and R oscillate between the lowest and the uppermost branches (the middle one being clearly unstable with respect to chemical diffusion). But they do not explain why R and C_s stay on a given branch as long as possible, nor does the theory describe the necessary up and down jumps. This points to a failure of the kinetic model which may be modified either by considering a discontinuity (analogous to phase changes or shock waves) or by adding a dynamic term (such as finite response time for one variable). Only the latter may lead to an oscillatory behaviour.

The important question is therefore: does the growth rate respond immediately to the changing composition? The advance of a solid-liquid interface is known, both theoretically^{22,23} and experimentally²⁴, to depend critically on the detailed structure of the interface on the molecular scale. As site formation is a probabilistic process, there will be a delay between a change in C_0 and a change in R . A plausible equation for R is thus:

$$\tau \frac{dR}{dt} + R = \mathcal{R}(C_0) \quad (4)$$

where τ , the characteristic time for structural rearrangement, is taken as constant for simplicity. Samoylovich²⁵ proposed a similar equation for the advance of a crystallization front with prescribed thermal gradients at the boundaries of the crystallizing region.

If τ is zero or steady-state obtains, equation (4) reduces to $R = \mathcal{R}(C_0)$, that is the growth rate is some function of the supersaturation which may be determined by laboratory experiments. According to what is known of nucleation and growth mechanisms, the positive function $\mathcal{R}(C_0)$ must vanish for $C_0 = C_L$ and increase with decreasing C_0 (at least for moderate supersaturations). Some indication of its magnitude and sensitivity to concentrations and temperature is provided by data from ref. 15. The essence of our analysis lies in equation (4) where we suggest that R should be considered as the solution of a differential rather than algebraic equation. Although this may represent a reasonable approach to growth mechanisms, it should be regarded as phenomenological. We show next that the proposed coupling between R and C_0 results in an oscillatory behaviour consistent with observations.

A quantitative model

Before dealing with equations (1) and (4), we describe qualitatively what happens during a typical episode of crystal growth. At time $t = 0$, growth starts in a regime controlled by the

interface kinetics. Initially the concentration profile in the melt phase is flat and C_0 , the concentration at the interface, is equal to C_m . As growth proceeds, a concentration boundary layer develops ahead of the crystal and growth becomes progressively diffusion controlled. As time increases, C_0 tends to C_L and the concentration profile tends asymptotically to that described by the boundary condition $C_0 = C_L$ (ref. 26, p. 146). Therefore, the boundary layer thickness δ behaves asymptotically as $\sqrt{t}$, and R behaves as $1/\sqrt{t}$. In this limit, the function $\mathcal{R}(C_0)$ in equation (4) may be approximated by an equation such as:

$$\mathcal{R}(C_0) = Q(C_L - C_0)^n \quad (5)$$

This general form of C_0 and R is sketched in Fig. 3. Throughout this evolution, the concentration boundary layer thickness δ increases as the crystal evolves towards higher and higher albite concentrations.

The situation is more complex for an oscillatory growing crystal. However, the observations show that the oscillations are of small amplitude and are superimposed on a regular evolution which follows along the lines described above. This will lead us to write the general solution as the sum of a regular slowly-varying solution and a perturbation of small amplitude. A remarkable aspect of the governing equations is that a qualitative knowledge of the regular solution enables us to predict the behaviour of the perturbations. We integrate equation (1) and obtain:

$$\int_0^\infty \frac{\partial C}{\partial t} dx = -D \left. \frac{\partial C}{\partial x} \right|_{x=0} + R(C_m - C_0) \quad (6)$$

We define a diffusion width $\xi(t)$, which is a measure of the thickness of the concentration boundary layer, as:

$$\int_0^\infty (C - C_m) dx = \xi(C_0 - C_m) \quad (7)$$

Using equation (7) and boundary conditions (2), equation (6) may be written as:

$$\frac{d}{dt} [\xi(C_0 - C_m)] = R(C_m - KC_0) \quad (8)$$

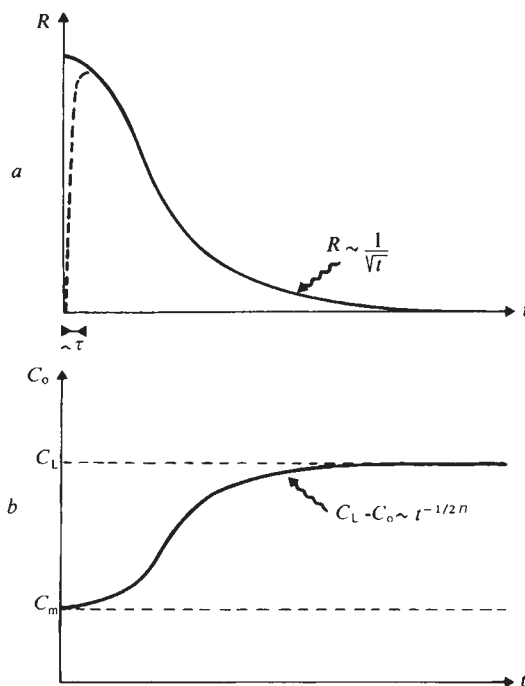


Fig. 3 Plots of the growth rate R (a) and the albite content of the melt at the interface C_0 (b) against time for a typical episode of crystal growth. When the conditions for oscillatory zoning are not fulfilled, it makes little difference whether there is a delayed response of the growth rate (dashed line in a) or not (solid line). n in b is the same as in equation (5).

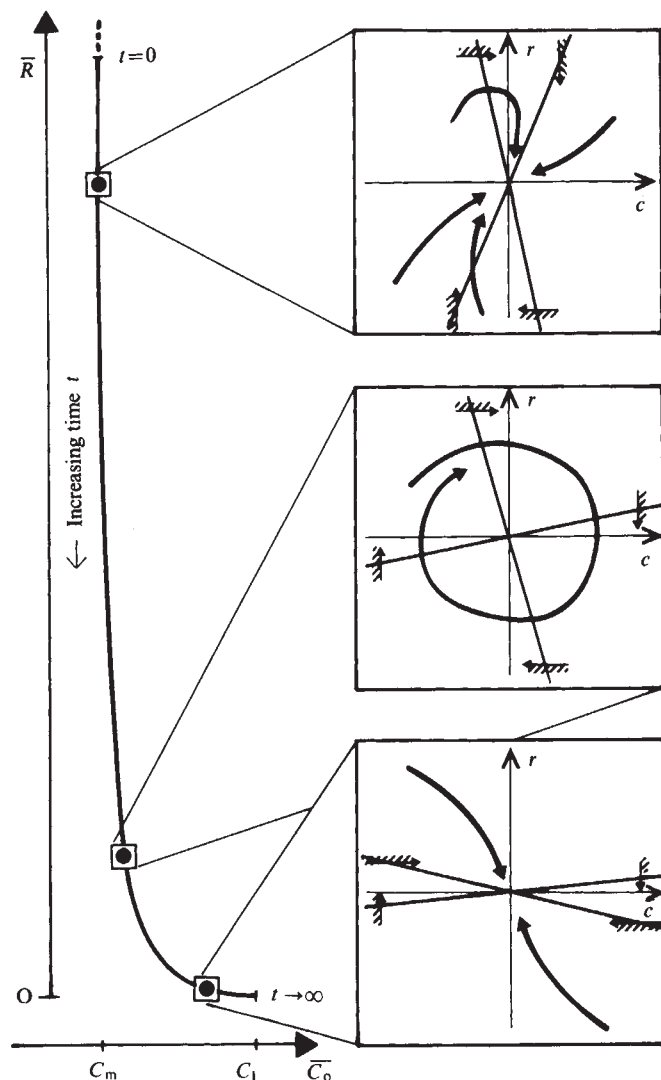


Fig. 4 Phase-plane representation of the solution $\{R, C_0\}$. The heavy curve on the left represents the evolution of the quasi-stationary regular solution $\{\bar{R}, \bar{C}_0\}$. Superimposed on that solution is a perturbation $\{r, c\}$ whose evolution with respect to the regular solution is shown in the three insets at three different stages of growth. In the insets, straight lines represent the isoclines $dc/dt = 0$ (equation (12)) and $dr/dt = 0$ (equation (13)), and arrows represent tangents to the evolution curves. We predict the perturbation $\{r, c\}$ to be rapidly and monotonically flattened out at both the beginning (upper inset) and the end (lower inset), but to exhibit damped oscillations (middle inset) over a finite time interval.

We now write the full solution $\{R, C_0\}$ as the sum of a slowly-varying regular solution $\{\bar{R}, \bar{C}_0\}$ and a perturbation of small amplitude $\{r, c\}$:

$$R = \bar{R} + r, \quad C_0 = \bar{C}_0 + c \quad (9)$$

The regular solution is described by the following equations:

$$\frac{d}{dt} [\xi(\bar{C}_0 - C_m)] = \bar{R}(C_m - K\bar{C}_0) \quad (10)$$

and

$$\tau \frac{d\bar{R}}{dt} + \bar{R} = \mathcal{R}(\bar{C}_0) \quad (11)$$

We assume next that ξ has the same value for both the full and the regular solution. This is not strictly valid for any kind of perturbation and essentially depends on the characteristic frequency of the oscillation when it exists. The perturbation is associated with a 'penetration depth' which increases as the

oscillation frequency decreases. In other words, the above assumption may be wrong if the oscillatory zoning period is greater than the characteristic time for crystal growth. Clearly, however, the zoning wavelength is much smaller than typical crystal dimensions. Neglecting second-order terms, we get:

$$\xi \frac{dc}{dt} + c \frac{d\xi}{dt} = \bar{R}(-Kc) + r(C_m - K\bar{C}_0) \quad (12)$$

and

$$\tau \frac{dr}{dt} + r = -\frac{\rho}{C_0} c \quad (13)$$

with

$$\rho = -\bar{C}_0 \left. \frac{d\mathcal{R}}{dC_0} \right|_{C_0=\bar{C}_0} \quad (14)$$

where the sign has been chosen so that ρ is positive, as $\mathcal{R}$ is a decreasing function of C_0 .

Equations (12) and (13) are the two perturbation equations to be solved. They are written with $\bar{R}$ and $\bar{C}_0$ as parameters and thus describe the perturbations r and c with respect to the quasi-stationary evolution of the system.

The system can be described qualitatively in the (R, C) phase plane (Fig. 4). There are two general types of trajectories in this plane: either a direct convergence or a spiral towards the regular point $\{\bar{R}, \bar{C}_0\}$. In terms of growth behaviour, this means either an ordinary convergence to the regular solution (normal growth) or damped oscillations around mean values $(\bar{R}, \bar{C}_0)$ (oscillatory zoning). These two types of behaviour are possible in different areas of the (R, C) plane and thus at different stages of growth.

We now discuss the system in a more quantitative way. The solutions $\{r, c\}$ of equations (12) and (13) represent either an exponential decay or damped sinusoidal oscillations depending on the sign of the discriminant:

$$\Delta = \left[\frac{1}{\tau} - \frac{1}{\xi} \left(K\bar{R} + \frac{d\xi}{dt} \right) \right]^2 - \frac{4\rho}{\xi\tau} \left(\frac{C_m}{\bar{C}_0} - K \right) \quad (15)$$

As K is < 1 , quantity $((C_m/\bar{C}_0) - K)$ is positive when growth begins ($\bar{C}_0 = C_m$) and remains positive if C_m is greater than C_s (we do not consider the 'hypersaturation' case $C_m < C_s$, which is more complex). As growth proceeds, $\bar{R}$ and $d\xi/dt$ decrease while ξ increases from 0 to ∞ . Therefore, the term in brackets in equation (15) increases monotonously from $-\infty$ to $1/\tau$, passing through zero when:

$$\xi = \tau \left(K\bar{R} + \frac{d\xi}{dt} \right) \quad (16)$$

The discriminant is thus positive when growth begins, becomes negative, and then positive again. This shows that oscillatory behaviour will happen at some intermediate stage of the crystal history (Fig. 4). The time t when equation (16) is verified marks the transition from the initial growth regime when growth rate is

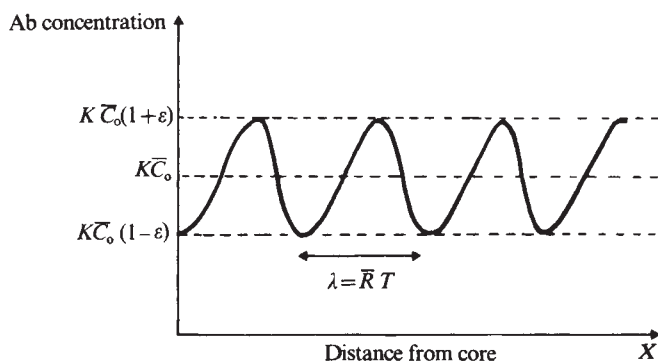


Fig. 5 When the required conditions are met (see text), the perturbation equations result in almost undamped, dissymmetric oscillations in the concentration profile.

essentially determined by the interface kinetics to the final regime when chemical diffusion is the main limiting factor.

However, oscillatory zoning will be detectable only if damping is not too large. The damping coefficient k is defined simply as the ratio of the concentration amplitude from one cycle to the next: k reaches a minimum $\hat{k}$ at a time close to $\hat{t}$ defined above. We find that damping is small ($\hat{k}$ close to unity) when at that time $\bar{R}/\rho$ is much lower than $((C_m/K\bar{C}_0) - 1)$. As shown by equation (14), this means a high sensitivity of the crystal interface to concentration changes. Consider, for example, equation (5) for $\mathcal{R}(C_0)$. This yields:

$$\frac{\bar{R}}{\rho} = \frac{1}{n} \left(\frac{C_L}{C_0} - 1 \right). \quad (17)$$

In this simplified case, oscillatory zoning is certainly detectable for very weak supersaturations as the low damping condition is fulfilled throughout the crystallization sequence.

Furthermore, a long oscillatory episode requires a large value for τ . This critical parameter is a measure of the difficulty of rearranging the crystal surface when composition changes. For the plagioclase system, the excess free energy of mixing exhibits large variations in the range $An_{30} - An_{90}$ because of a complex substitution process due to a transition between a disordered 7 Å and an ordered 14 Å crystal framework (ref. 25, p. 135).

In these conditions, low amplitude, almost undamped, oscillatory zoning is described by:

$$C_s(t) = K\bar{C}_0 (1 + \varepsilon \sin \omega t)$$

compared with interface position:

$$X(t) = X(0) + \bar{R}t + \varepsilon \frac{\rho}{\tau \omega^2} \sin \omega t \quad (18)$$

where ε is the perturbation amplitude. Albite concentration is thus a dissymmetric periodic function of distance X , decreasing more abruptly than it rises (Fig. 5), in agreement with observations^{6,7}. The higher the perturbation amplitude ε , the more pronounced the dissymmetry.

The above discussion has clarified the conditions for oscillatory behaviour, which seems to be a delicate phenomenon most likely when growth rates are small. We now give numerical estimates for the various parameters.

Equation (1) may be approximated by:

$$D \frac{\partial^2 \bar{C}}{\partial x^2} + \bar{R} \frac{\partial \bar{C}}{\partial x} = 0 \quad \text{in a region } x < x_0 \text{ (close to the interface)}$$

and by

$$D \frac{\partial^2 C}{\partial x^2} = \frac{\partial C}{\partial t} \quad \text{in a region } x > x_0 \text{ (far from the interface)}$$

This shows that a reasonable approximation to the concentration profile close to the interface is:

$$\bar{C} = C_m + (\bar{C}_0 - C_m) \cdot \exp\left(-\frac{\bar{R}}{D} \cdot x\right)$$

Thus $D/\bar{R}$ is a reasonable estimate for ξ . We now summarize the quantitative aspects of our theory. We denote by T the oscillation period. The zoning wavelength λ is equal to $\bar{R}T$. The damping factor k is taken as $\hat{k} = \exp(T/\tau)$ and small damping implies that ratio T/τ is small. This leads to the constraint:

$$\tau \left(K\bar{R} + \frac{d\xi}{dt} \right) \approx \xi = \frac{D}{\bar{R}}$$

Making the further approximation that $d\xi/dt \ll \bar{R}$, we obtain the following set of conditions:

$$\lambda = \bar{R}T = 10 \mu\text{m}; \quad \bar{R}^2\tau = \frac{D}{K} \approx 10^{-8} \text{ cm}^2 \text{ s}^{-1} \quad (\text{refs 6, 28})$$

and T/τ small (< 0.1 , say).

The numerical analysis requires a value for the growth rate in realistic conditions. The only measurements available are those

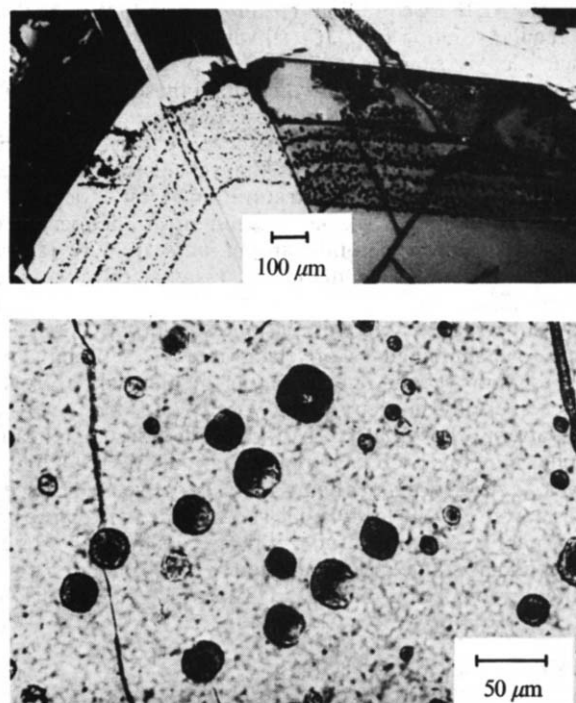


Fig. 6 Rounded glassy inclusions within two different oscillatory zoned plagioclases from North Atlantic tholeiitic basalts. Note that, despite the presence of inclusions, oscillatory zones remain remarkably straight and parallel (photographs from ref. 12).

of Lofgren¹⁵ who made plagioclase growth experiments at comparatively low temperatures (800–1,000 °C) and over rather short times (between 10 and 70 h). Taking, for example, a value of $10^{-7} \text{ cm s}^{-1}$, a value actually measured by Lofgren¹⁵, we obtain:

$$\tau = 10^6 \text{ s} \quad \text{and} \quad T/\tau = \ln k = 10^{-2}$$

A value of $10^{-6} \text{ cm s}^{-1}$ for the growth rate would yield the following estimates:

$$\tau = 10^5 \text{ s} \quad \text{and} \quad T/\tau = 0.1$$

The characteristic time τ remains poorly constrained but is probably quite large. It may depend on the degree of supersaturation and the estimates quoted above are only valid in the low supersaturation limit.

Discussion

Competition between chemical diffusion and interface kinetics should affect all crystals which grow from a melt which is not pure. Delayed response of the growth rate to concentration changes should also be universal. The growth equations predict an episode of oscillatory zoning for all systems provided that the supersaturation is not too large ($C_s < C_m < C_L$). This may seem strange as oscillatory zoning seems to be the exception rather than the rule. Of course, equation (4) is only one way that a time delay could express itself but the explanation is more likely to lie in the numerical values of the characteristic parameters involved in practical situations. For a system in given (C_m , temperature) conditions, oscillatory behaviour is predicted over a certain interval Δt around a time $\hat{t}$ which marks the transition between interface- and diffusion-controlled growth. For most systems this transition happens much too early or much too late to be detected practically with the result that these systems are classified as interface- or diffusion-controlled. Also Δt must be large enough for oscillations to occur on a measurable crystal length. The oscillation period T must be small enough for the transition episode to include a reasonable number of oscillations and large enough for the zonations, of wavelength $\lambda = \bar{R}T$, not to be erased by subsequent solid diffusion. A precise

investigation of these conditions requires a quantitative analysis of the regular solution $\{\bar{R}(t), \bar{C}_0(t)\}$ which is beyond the scope of this article. We simply suggest that the plagioclase-melt system is one of the rare systems which fulfill these requirements.

But we have also shown that these requirements were not sufficient: for oscillatory behaviour to be more than an academic prediction, the damping coefficient k must be close to unity, and this in turn requires that the supersaturation be low enough. This explains why oscillatory zoning is common in phenocrysts of volcanic rocks and apparently absent or extremely rare in laboratory experiments²⁹. Only when a magma body cools very slowly can plagioclase crystals come to an appreciable size in very low supersaturation conditions. Our quantitative model is certainly oversimplified. More laboratory experiments are needed to improve our understanding of plagioclase growth kinetics, especially in view of the variety and complexity of zoning patterns. That plagioclases are often more complicated than other minerals probably implies that they record a longer history, long residence times being allowed for even in quiescent magmas because of the low plagioclase-melt density contrast³⁰.

Remarkably, when oscillatory zoning occurs the planar interface is extremely stable. This is attested by an observation made by Bollinger¹² on oceanic basalts: in these, small glass inclusions

are sometimes trapped by the growing crystal but do not lead to the formation of dendrites, showing that these lateral perturbations are stabilized (Fig. 6). Such a stability is expected for low supersaturations^{31,32}. However, a few samples show contorted bands not conforming to prominent faces¹¹. It would be interesting to investigate how the time delay in equation (4) affects the stability of the interface shape.

Conclusions

Our new theory explains the phenomenon of oscillatory zoning which is observed in many naturally grown plagioclase crystals. The theory is based on a simple model of the interface kinetics sensitivity to concentration changes in the melt. The mechanism suggested is probably very general but its effects can only be observed in specific conditions. We believe that an oscillatory zoned plagioclase is a pathological case of crystal growth which enables us to study a fundamental process, that is the response of the crystal growth rate to concentration changes. This process may be responsible for some other characteristics of crystal growth which remain poorly understood, such as the stability of a planar interface.

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Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumour virus chimaeric plasmids

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Fusions between the mouse mammary tumour virus long terminal repeat and a mouse dihydrofolate reductase cDNA have been constructed in a SV40 vector. When these plasmids are transferred into recipient cells, the production of dihydrofolate reductase is regulated by glucocorticoid hormones. These results define a hormonally responsive region of the viral genome.

ALL steroid hormones seem to function largely via a common molecular mechanism in which the hormone first associates with a specific soluble receptor protein present in target tissues^{1,2}. This interaction causes an alteration in the receptor that increases its affinity for binding sites in the cell nucleus^{3,4}. The net effect of steroid action is to alter the pattern of proteins synthesized by the cell, probably by altering the transcription of a few specific genes⁵. It is widely documented that steroid-receptor complexes have DNA-binding properties² and that nuclear translocation reflects association of the complexes with

DNA⁶. Furthermore, both genetic and biochemical evidence has suggested that DNA binding may be involved in eliciting a biological response⁷. To date, however, there is no direct evidence that interactions with DNA lead to changes in transcription or that steroid-receptor complexes must interact with particular DNA sequences to alter gene expression.

We have used mouse mammary tumour virus (MMTV)-infected cells as a model to study the regulation of specific gene expression by steroid hormones⁸. When integrated into host cell DNA, the viral genome (the provirus) is transcribed by cellular RNA polymerase II⁹ and production of viral RNA is stimulated by glucocorticoid hormones such as dexamethasone¹⁰. Several

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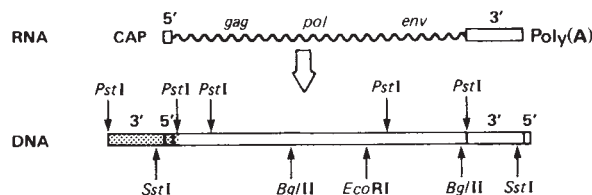


Fig. 1 The structure of the RNA and DNA forms of the MMTV genome. The RNA (~9 kilobase [kb]) form is shown with the putative coding regions indicated: *gag*, group-specific antigens, core proteins; *pol*, viral reverse transcriptase; *env*, viral envelope glycoprotein; *?*, a postulated but unidentified gene product¹⁴. The linear form of MMTV proviral DNA is shown below; on integrating into cell DNA, this structure (with minor modifications at the ends) is maintained. At each end is a LTR consisting of sequences coding for both the 5' and 3' ends of viral RNA; these are shown as the boxes with the structure 3' (~1,200 bp)/5' (130 bp). The *Pst*I fragment from the left-hand end (shown stippled) contains all but a few base pairs of one LTR in addition to 135 bp coding for RNA beyond the 5' leader or 'strong-stop' sequence. This *Pst*I fragment was used as the MMTV fragment in construction of the plasmids shown in Fig. 2.

lines of evidence suggest that hormonal stimulation of MMTV RNA synthesis is mediated by the same intracellular glucocorticoid receptors that are involved in regulation of cellular genes¹¹. Like other retroviruses, MMTV DNA integrates at many sites, perhaps even randomly, in the host genome¹². Studies of viral RNA production in many different clones of MMTV-infected cells suggest that hormonal regulation of MMTV gene expression is not site specific; indeed, there are multiple cellular sites where the provirus is under hormonal control¹². This argues against the hypothesis that regulation of viral gene expression occurs as a result of integration into a hormonally regulated site on the host chromosome.

In the present studies we sought to determine whether MMTV encodes its own promoter as well as a region required for glucocorticoid responsiveness. Our approach is based on one used successfully in the study of bacterial gene regulation, entailing the construction of a gene fusion between the regulatory region from the gene of interest and the coding region of a second gene whose activity can be conveniently assayed and whose function can be selected for *in vivo*. The expression of the fused gene may then be subject to the control signals present on the regulatory region.

As with other retroviruses, MMTV proviral DNA is bounded by two identical regions composed of sequences derived from both the 5' and 3' ends of the viral genome. These regions are called long terminal repeats (LTRs) and in MMTV are ~1,200 base pairs (bp) long (Fig. 1). The location of the 5' end of MMTV RNA corresponds to a site represented by the junction of the 3' and 5' sequence in the left LTR (Fig. 1), suggesting that the promoter for viral transcription lies within this 1,200-bp region. We have constructed fusions between the MMTV LTR and a cDNA containing the coding region for mouse dihydrofolate reductase (DHFR); the expression of this enzyme can be selected for in the appropriate host cell. These fusions were constructed in a plasmid DNA vector which allows expression of inserted genes after these molecules are introduced into recipient cells¹³. Analyses of the expression and hormonal regulation of these recombinant molecules have allowed us to identify the LTR of MMTV as a region which confers glucocorticoid responsiveness.

Construction of MMTV-*dhfr* recombinant plasmids

The *Pst*I fragment from the left end of the viral DNA encompasses an entire LTR (except for 5–10 bp at the extreme left-hand end). The cap site on the 5' end of MMTV RNA is located ~270 bp from the right-hand end of the *Pst*I fragment (ref. 14 and J. Majors and H. Varmus, personal communication); transcription proceeds in the right-hand direction (Fig. 1). It therefore seemed probable that both the promoter for viral RNA synthesis and the sequences involved in hormonal regulation are located within this fragment. We therefore converted the ends of

this 1,450-bp fragment (see Fig. 2 legend) by addition of *Hind*III 'linkers' to facilitate its insertion into the unique *Hind*III site of the vector pSV2 δ hfr (Fig. 2a). This is a hybrid vector composed of sequences from the papovavirus SV40, the *Escherichia coli* plasmid pBR322 and cDNA coding for the mouse DHFR¹⁵. Sequences derived from pBR322 allow cloning and propagation of the plasmid in *E. coli* while those derived from SV40 contain processing signals allowing production of a functional messenger RNA for DHFR within mammalian cells¹⁶.

Individual colonies harbouring the resulting plasmid were screened to identify molecules containing the MMTV fragment oriented in the same direction as the DHFR-coding region. Such a configuration of the MMTV-*dhfr* fusion would allow transcription initiating at a promoter within the viral fragment also to transcribe *dhfr*. This plasmid, designated pSVMdhfr, is shown in Fig. 2b. In addition, a derivative of pSVMdhfr, constructed by deleting the SV40 fragment which contains the early promoter (Fig. 2c) and called pMTVdhfr, retains the putative MMTV promoter and regulatory region.

Transformation characteristics of *dhfr*-containing plasmids

Dihydrofolate reductase catalyses the formation of tetrahydrofolate, a cofactor essential for many one-carbon transfer reactions such as in the biosynthesis of glycine and thymidine. A cell line¹⁷ derived from Chinese hamster ovary (CHO) cells provides an ideal recipient for the *dhfr* vectors in DNA transformation experiments. These cells are defective in DHFR and, unlike wild-type cells, are unable to grow in medium lacking glycine,

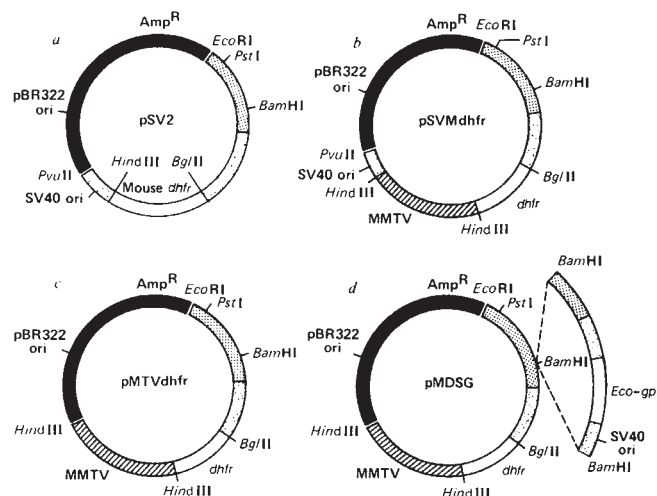


Fig. 2 Structure of plasmid vectors containing mouse *dhfr* and *E. coli* *gpt*. The solid black segment of each plasmid is a 2.3-kb fragment of pBR322 extending from the *Eco*RI site to the *Pvu*II site which contains the lactamase gene (ampicillin resistance) and the origin of replication. The lightly stippled region of pSV2, pSVM and pMDSG between the *Pvu*II and *Hind*III sites is a 325-bp fragment of SV40 (coordinates 0.65–0.71) containing the early promoter and the SV40 origin of replication. The other stippled regions are SV40 sequences encoding the intron for small T antigen (lightly stippled, SV40 coordinates 0.44–0.56) and the site for polyadenylation for early mRNA (heavily stippled, SV40 coordinates 0–0.19). All are described in ref. 13. The hatched region on pMTV, pSVM and pMDSG is the MMTV LTR. The open regions are either mouse *dhfr* cDNA¹⁵ or *E. coli* *gpt*¹³. Transcription from both the SV40 early promoter and the MMTV promoter is in the anticlockwise orientation, as are the coding sequences of *dhfr* and *gpt*. The sizes of the fragments are drawn only approximately to scale. Derivatives of pSV2 δ hfr were constructed as follows. The 1.4-kb *Pst*I MMTV LTR fragment was treated with T4 DNA polymerase to remove protruding ends, then *Hind*III linker fragments were added by ligation with T4 ligase. This fragment was inserted at the unique *Hind*III site of pSV2 δ hfr separating the SV40 origin fragment from *dhfr* sequences to produce pSVMdhfr (b). pMTVdhfr (c) was generated by digesting pSV2 δ hfr with *Pvu*II and *Hind*III, adding *Hind*III linkers to the *Pvu*II site and inserting the modified MMTV fragment. Colonies were screened to isolate molecules containing the MMTV DNA in the appropriate orientation. pMDSG (d) was constructed by inserting a 2.2-kb *Bam*HI fragment containing the SV40 origin/promoter fragment fused to *Eco*-*gpt* and SV40 RNA processing signals into the unique *Bam*HI site of pMTVdhfr. This 2.2-kb fragment was derived by addition of a *Bam*HI linker to the *Pvu*II site of pSV2 δ gpt¹³.

Table 1 Transformation efficiencies and hormonal regulation of *dhfr*-containing plasmids in *dhfr*⁻ CHO cells

Vector	Structure	Transformation frequency	Hormonal regulation
pSV2dhfr	SV40- <i>dhfr</i>	$1-2 \times 10^{-4}$	— (0/4)
pSVMdhfr	SV40-MTV- <i>dhfr</i>	1×10^{-4}	+
pMTVdhfr	MTV- <i>dhfr</i>	1×10^{-6}	+
pMDSG	MTV- <i>dhfr</i> :SV40- <i>gpt</i>	1×10^{-4}	+
pSVMdhfr'	SV40-VTM- <i>dhfr</i>	$1-2 \times 10^{-5}$	— (0/4)
pSV0dhfr	<i>dhfr</i>	$\leq 1 \times 10^{-7}$	Not determined

Transformations were performed using the calcium phosphate co-precipitation method of Graham and van der Eb¹⁸. In brief, 10–15 µg of circular plasmid DNA were precipitated with CaPO₄ and added to 10⁶ cells growing in non-selective medium (Ham's F12 medium). Four hours after addition of DNA, cells were 'glycerol shocked' with 20% glycerol in phosphate-buffered saline (PBS) according to the method of Frost and Williams²⁵. Medium was replenished the following day and the cells allowed to grow for 2 more days in non-selective conditions. Cells were then passaged into selective medium (Dulbecco's minimal essential medium + 34.5 µg ml⁻¹ proline); 10⁶ cells were plated per 10-cm dish. Medium was changed every 2–3 days and *dhfr*⁺ clones became visible 7–10 days later. Transformation frequencies are reported as transformants per cell (for example, 100 colonies on a dish plated with 10⁶ cells yields a frequency of 10⁻⁴ transformants per cell). Hormonal regulation was determined either by methotrexate resistance (Fig. 3) or ³H-methotrexate binding (Table 2); the numbers in parentheses indicate the number of individual transformants analysed and the proportion which were hormonally responsive. In pSVMdhfr', VTM indicates that the MMTV fragment was inserted in the anti-sense orientation.

thymidine and hypoxanthine. One can identify clones expressing the *dhfr* gene present on the transforming DNA by selecting for clones able to grow in the absence of one or more of the required supplements.

DNA was introduced into the CHO *dhfr*⁻ cells using the transformation procedure of Graham and van der Eb¹⁸. A calcium phosphate precipitate containing 15 µg of plasmid DNA was added to 10⁶ cells growing in non-selective medium; after transfer into selective medium, colonies were seen within 7–10 days. When pSV2dhfr DNA was used we observed 100–200 colonies (frequency $\sim 1-2 \times 10^{-4}$ per cell). Similar results have been obtained by Subramani *et al.*¹⁶. The expression of *dhfr* in these cells is apparently directed by the SV40 early promoter because very few transformants (frequency $\leq 1 \times 10^{-7}$ per cell) are observed if transformations are attempted with a plasmid (pSV0dhfr) lacking the SV40 promoter/origin fragment (Table 1). pSV0dhfr was generated by deleting the region between the *Pvu*II and *Hind*III sites of pSV2dhfr (Fig. 2a).

Similar experiments were performed with pSVMdhfr and pMTVdhfr. As shown in Table 1, the frequency of transformation with pSVMdhfr is similar to that with pSV2dhfr. The possibility exists, however, that the SV40 early promoter, located directly upstream of the MMTV LTR, is responsible for *dhfr* expression by readthrough transcription. This could occur with or without removal of MMTV sequences via a post-transcriptional splicing event. Results obtained with the pMTVdhfr vector eliminate this possibility because the SV40 promoter is absent. When CHO *dhfr*⁻ cells were infected with this DNA, transformants were indeed obtained, although at a considerably reduced frequency ($\sim 10^{-6}$ per cell; Table 1). Nevertheless, the ability to isolate *dhfr*⁺ colonies following transformation with pMTVdhfr suggests that sequences present on the MMTV LTR are serving a promoter-like function; this is supported by studies of the 5' end of the *dhfr* RNAs in the transformants (see below).

We considered whether pMTVdhfr is less efficient at producing *dhfr*⁺ colonies because the MMTV promoter is intrinsically inefficient. Such a weak promoter might produce levels of *dhfr* RNA only marginally sufficient to support growth in selective medium, thereby leading to reduced numbers of transformants. Thus we constructed another vector derived from pMTVdhfr in which the MMTV-*dhfr* fusion could be introduced into cells without directly selecting for *dhfr* expression; DHFR production might be detectable in the CHO *dhfr*⁻ cells even if those levels were not sufficient to support growth. This vector, designated pMDSG (Fig. 2d), contains a DNA fragment that encodes the *E. coli* xanthine-guanine phosphoribosyl transferase (*gpt*)

gene under SV40 early promoter control inserted at the *Bam*HI site of pMTVdhfr. The expression of the *E. coli* gene can be selected for in the CHO *dhfr*⁻ cells using the dominant selection procedure described by Mulligan and Berg¹³. The frequency of *gpt*⁺ clones obtained with pMDSG approximates the frequency of *dhfr*⁺ transformants obtained with pSV2dhfr or pSVMdhfr (1×10^{-4} per cell). Three individual clones were picked, grown to mass culture and transferred to DHFR-selective medium. Surprisingly, all three clones grew as well as other *dhfr* transformants (data not shown). Furthermore, when pMDSG was used to transform cells selecting for *dhfr* directly, transformants also arose at a frequency of $\sim 1 \times 10^{-4}$ per cell (Table 1). The relatively high level of transformation with pSVMdhfr and pMDSG, compared with that with pMTVdhfr, seems to be due to the presence of the SV40 fragment encompassing the promoter and origin of replication (S. Subramani and P. B. M. Fromm and P.B., F.L. and G.R., unpublished observations). This novel effect of the SV40 promoter/origin region on the efficiency of DNA mediated transformation will be described in greater detail elsewhere.

Expression and glucocorticoid regulation of *dhfr* in transformants

Regardless of differences in transformation frequencies, our results suggest that the *dhfr* cDNA can be expressed when fused to the MMTV LTR. This would be consistent with the presence of a promoter on this fragment of MMTV DNA, perhaps near the cap site. Our major interest in these experiments was to ascertain whether the expression of *dhfr* in these MTV-*dhfr* fusions could be regulated by glucocorticoids. A positive answer would suggest that the viral DNA indeed carries its own regulatory site.

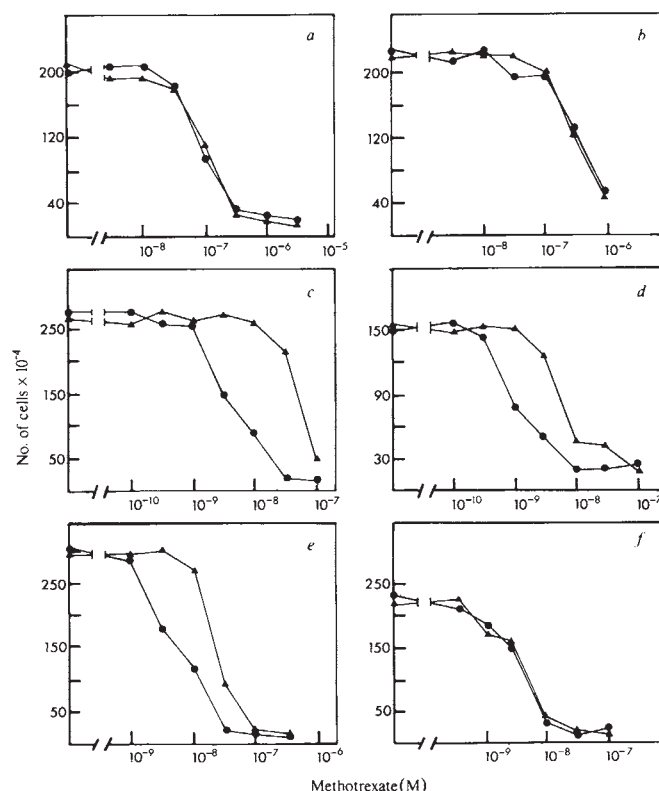


Fig. 3 Methotrexate inhibition of CHO *dhfr*⁻ cells transformed with *dhfr* cDNA-containing plasmids. Individual clones of CHO *dhfr*⁻ cells arising from transformation with the indicated plasmids were grown in selective medium (Dulbecco's minimal essential medium + 39.5 µg ml⁻¹ proline). In each case, $\sim 10^5$ cells were plated on 6-cm dishes either in the presence or absence of 10^{-6} M dexamethasone. Twenty-four hours later, methotrexate was added to individual dishes at the indicated concentrations. Fresh medium containing methotrexate was added after 2 days and the surviving cells counted after 2 more days. *a*, Wild-type CHO1 cells; *b*, transformant pSV2dhfr.1; *c*, transformant pMTVdhfr.2; *d*, transformant pMDSG.8; *e*, transformant pSVMdhfr.4; *f*, transformant pSVMdhfr'.6. Dexamethasone absent (●) or present (▲).

Table 2 ^3H -methotrexate binding of cell extracts

Transformant	c.p.m. ^3H -methotrexate bound		Fold induction
	-Dex	+Dex	
pSV2dhfr.1	9,882	8,745	0
pMTVdhfr.1	116	571	5
pMTVdhfr.2	444	1,148	3
pMTVdhfr.3	972	5,111	5

Cells of individual clones were grown in Dulbecco's minimal essential medium + proline in the presence or absence of 10^{-6} M dexamethasone (Dex) for 2 days. Cells were removed from dishes with PBS/EDTA and washed once with PBS. $1-2 \times 10^7$ cells were resuspended in 1 ml of 0.05 M potassium phosphate buffer pH 7.4. The suspended cells were frozen and thawed three times in a dry ice/ethanol bath, and then centrifuged for 15 min in a microfuge. ^3H -methotrexate (Amersham, 200 mCi mmol $^{-1}$) was added to equal amounts of extract from each cell line (~ 300 μg total protein in 50 μl) and allowed to bind in the dark for 10 min at 25 $^{\circ}\text{C}$. The mixture was passed over an 8-ml Sephadex G-50 column equilibrated in 0.01 M KPO_4 pH 6, 0.15 M KCl. The counts in the excluded volume were determined by counting in a scintillation spectrometer. In subsequent experiments we observed four- to fivefold inductions in all three pMTVdhfr clones.

Initially we attempted to measure DHFR in pMTVdhfr transformants using a standard enzymatic assay which measures conversion of ^3H -folate to ^3H -dihydrofolate and tetrahydrofolate. However, the levels of the enzyme are too low to be detected reproducibly in these cells using such an assay (F.L. and G.R., unpublished results). We therefore used methotrexate, a folate analogue which inhibits DHFR and thus inhibits cell growth, to estimate levels of the enzyme. Cells were plated in various concentrations of methotrexate, in either the presence or absence of dexamethasone, and the number of cells on each dish determined 4–5 days later. Figure 3a shows the results of such an experiment using wild-type CHO-K1 cells. The concentration of methotrexate required to inhibit growth of these cells by 50% is $\sim 10^{-7}$ M; dexamethasone has no effect on the cells' sensitivity to methotrexate. Similar results are observed when pSV2dhfr transformants of the CHOdhfr $^{-}$ cells are examined (Fig. 3b). Again, methotrexate sensitivity is unaffected by hormone treatment; this is true of four independent pSV2dhfr transformants we have examined.

In contrast, in experiments using pMTVdhfr transformants, the cells were considerably more resistant to methotrexate in the presence of dexamethasone than in its absence (Fig. 3c); the hormone-treated cells are resistant to ~ 10 – 20 -fold more methotrexate. Furthermore, the levels of methotrexate required to inhibit growth of these cells (10^{-9} – 10^{-8} M) is lower than that required to inhibit pSV2dhfr transformants. We believe this indicates that the MMTV promoter is less efficient than either the SV40 early or the cellular *dhfr* promoter.

In cells transformed with pMDSG DNA, dexamethasone also increases the resistance to methotrexate (Fig. 3d). Similar results have been obtained with several clones of pSVMdhfr transformants (Fig. 3e). Indeed, all cells transformed with a plasmid containing *dhfr* cDNA fused to the MMTV LTR in the proper orientation exhibit an approximate 10-fold increase in methotrexate resistance when grown in the presence of dexamethasone. If, however, we use a vector in which the MMTV LTR is inserted into pSV2dhfr in the opposite orientation relative to the *dhfr* cDNA (pSVMdhfr'), resultant transformants do not respond to dexamethasone (Fig. 3f). We do not know whether the *dhfr* expressed in these cells arises from a promoter on the opposite strand of the MMTV LTR. Nevertheless, the results of all the experiments indicate that the MMTV LTR is required for hormonal regulation of *dhfr* and that it must be in the proper orientation (Table 1).

Previous studies¹⁹ have indicated the lack of a 1:1 correlation between the amount of DHFR present in a cell and a cell's sensitivity to methotrexate. To obtain a more quantitative estimate of the levels of DHFR in the transformed cells, we have measured ^3H -methotrexate binding to DHFR in cell extracts. Table 2 summarizes the results of such an experiment. pMTVdhfr-transformed cells clearly contain three- to fivefold more DHFR when grown in the presence of dexamethasone than in its absence; as described above, this results in a 10–20-fold increase in methotrexate resistance. Cells transformed with

pSV2dhfr contain higher levels of DHFR; however, these levels are unaffected by the hormonal status of the cell. Similar experiments with extracts from pMDSG- and pSVMdhfr transformants indicate that glucocorticoids result in a consistent three- to fivefold increase in levels of DHFR. All the data are in agreement with the methotrexate growth-inhibition studies.

Note that the level of induction of DHFR in the CHO cells is somewhat lower than the average 10-fold induction of MMTV RNA in mouse mammary tumour cells and much lower than the 50–500-fold level of induction of MMTV RNA in infected HTC, rat hepatoma cells. The reasons for these differences are obscure, although we have observed that the concentration of glucocorticoid receptors in the CHO cells is ~ 5 – 10% that of HTC cells. We are investigating whether the induction of *dhfr* or other gene fusions can be augmented in cells with higher levels of receptor. Indeed, recent experiments in mouse 3T6 cells using pSVM plasmids containing the *Eco*-*gpt* gene rather than *dhfr* cDNA, indicate that production of xanthine-guanine phos-

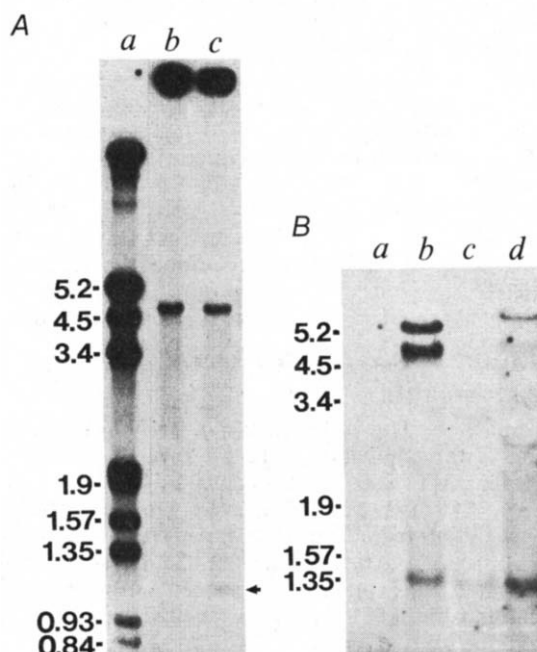


Fig. 4 A, S_1 nuclease mapping of MTV-*dhfr* RNA made in transformant pMTVdhfr.2. The ^{32}P -end-labelled probe for the hybridization was prepared as follows. pMTVdhfr plasmid was cut at the unique *Bgl*II site at the end of the *dhfr* cDNA (see Fig. 2). After treatment with alkaline phosphatase and labelling the 5' ends with ^{32}P -ATP and T4 polynucleotide kinase, the linear DNA was digested with *Eco*RI. The two resulting fragments were run on an agarose gel and the 4.5-kb *Eco*RI-*Bgl*II fragment containing the MMTV LTR and *dhfr* cDNA labelled at the *Bgl*II end was isolated from agarose. Total cytoplasmic RNA was isolated from pMTVdhfr.2 cells grown with or without dexamethasone (10^{-6}) as described previously²⁶. Poly(A)-containing RNA was isolated by binding to oligo(dT) cellulose. Approximately 5 μg of poly(A)-containing RNA was hybridized with an excess of end-labelled DNA fragment in 80% formamide according to the method of Berk and Sharp²⁰. Hybridization was performed at 50 $^{\circ}\text{C}$ for ~ 16 h. Hybrids were treated with S_1 nuclease (Boehringer-Mannheim) and run on a 1.4% agarose gel which was autoradiographed for 7 days. a, ^{32}P -end-labelled λ DNA (digested with *Eco*RI and *Hind*III; sizes are indicated in kb); b, MTVdhfr.2 RNA minus dexamethasone; c, MTVdhfr.2 RNA plus dexamethasone. The arrow indicates the ~ 1 -kb RNA-DNA hybrid. The band in b and c at ~ 4.5 kb is the reannealed *Eco*RI-*Bgl*II probe. The dark band at the top of b and c represents material that did not enter the gel. B, Analysis of pMTVdhfr DNA in three *dhfr* $^{+}$ clones. Three independent *dhfr* $^{+}$ clones arising following transformation with pMTVdhfr DNA were picked and grown to mass cultures. Total cell DNA was prepared from each clone as well as from the parental CHOdhfr $^{-}$ cells. 10 μg of each DNA was digested to completion with *Hind*III and run on a 0.8% agarose gel, and then transferred to nitrocellulose by the method of Southern²². The filter was hybridized with $\sim 5 \times 10^6$ c.p.m. of ^{32}P -nick-translated pMTVdhfr DNA, washed and autoradiographed as described previously¹². Order of samples: a, CHOdhfr $^{-}$; b, MTVdhfr.1; c, MTVdhfr.2; d, MTVdhfr.3. The molecular weight markers were unlabelled fragments of DNA cut with *Eco*RI and *Hind*III, visualized by ethidium bromide staining. The common band appearing in all the MTV *dhfr* clones, ~ 1.4 kb long, is the MMTV LTR fragment which is excised by *Hind*III (see Fig. 2).

phoribosyl transferase is augmented at least 10–20-fold by dexamethasone (F.L. and G.R., unpublished results).

Moving the 5' end(s) of MMTV-*dhfr* RNA

To determine whether the RNA expressed in the pMTVdhfr transformants initiates within MMTV DNA, we analysed the 5' end of the transcripts by the procedure of Berk and Sharp²⁰. The *Bgl*II-*Eco*RI fragment encompassing the MMTV-*dhfr* sequences of pMTVdhfr was labelled with ³²P at the *Bgl*II site. Hybrids between this fragment and poly(A)⁺ RNA from the cytoplasm of pMTVdhfr.2 cells were treated with S₁ nuclease and analysed by electrophoresis in 1.4% agarose gels. The hybrid band detected by autoradiography is ~1,000 bp long (Fig. 4a), indicating that the predominant 5' end of *dhfr* RNA maps to a site within the MMTV LTR, 250–275 bases upstream of the *dhfr* insert. Furthermore, the production of this RNA is stimulated in cells treated with dexamethasone.

The S₁ nuclease analysis shown in Fig. 4a contains a heterogeneous background of apparent hybrids 1.0–4.5 kbp long. These seem to be artefacts of incomplete S₁ digestion rather than random sites of initiation; the long exposure time required to see the hybrid band has made it technically difficult to overcome this problem. However, transformants grown in progressively increasing concentrations of methotrexate amplify the MMTV-*dhfr* fusion and overproduce the *dhfr* RNA several hundredfold²¹. S₁ nuclease analysis of RNA from such cells indicates that >90% of the *dhfr* RNA molecules initiate at the same site within the MMTV LTR as that shown by the 1-kilobase band in Fig. 4a²¹. In these conditions the background is eliminated because much shorter exposure times (4 h compared with 7 days) are required to visualize the authentic hybrid.

Integration of pMTVdhfr DNA in transformants

To determine the fate of the plasmid DNA used for transformation of the CHOdhfr⁻ cells, we have analysed cellular DNAs from pMTVdhfr transformants by the procedure of Southern²². Cell DNAs were cleaved with *Hind*III, which cleaves pMTVdhfr twice, and the fragments resolved by agarose gel electrophoresis. After transfer of the DNA onto nitrocellulose filters, the plasmid sequences were detected by hybridization with pMTVdhfr DNA labelled with ³²P by nick-

translation. As seen in Fig. 4b, the three independently isolated transformants harbour plasmid-related sequences in different locations within cell DNA, as judged by the different mobilities of the hybridizing bands. This is corroborated by analysis of DNAs digested with *Eco*RI (data not shown).

We have not yet mapped the sites of integration either within the host or the plasmid DNAs. Interestingly, in this limited sample of clones there seem to be only one to two copies of integrated vector DNA in the transformants. We have no evidence of free plasmid DNA being maintained within the pMTVdhfr-transformed CHO cells.

Conclusions

The results of the experiments described here provide evidence that the MMTV LTR contains sequences which are sufficient for glucocorticoid regulation of gene expression. This extends the observations of others that the entire MMTV genome is glucocorticoid sensitive when introduced into mouse L cells by DNA-mediated transformation^{23,24}. Whether glucocorticoids are acting by regulating transcription *per se* remains to be established. The LTR fragment we have used contains ~270 bp which encode the 5' end of MMTV RNA, and differences in RNA processing within this region may account for hormonal regulation of MMTV RNA production in virus-infected cells or of *dhfr* in the transformants described here. In addition, there may be other viral sequences outside the LTR or cellular sequences which contribute to the hormonal response. Whatever the mechanism(s), we are now in a position to determine which sequences within this MMTV fragment are required for hormonal responsiveness. It will be of interest to determine whether these sequences serve as specific recognition sites for the glucocorticoid-receptor complex.

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Hexose transport in hybrids between malignant and normal cells

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The kinetic parameters of hexose uptake were measured in matched pairs of hybrids between malignant and normal cells. Each pair consisted of a hybrid in which malignancy was initially suppressed and a segregant tumour derived from it. Comparisons were also made between tumour cells and non-tumorigenic derivatives selected from the tumour cell populations in vitro. Without exception, malignancy, as defined by the ability of the cell to grow progressively in vivo, was found to be linked to a systematic decrease in the Michaelis constant of the hexose transport system.

FOLLOWING the initial observation of Hatanaka *et al.*¹, many authors have reported that morphological transformation of fibroblastic cells *in vitro* by certain oncogenic viruses is associated with an increase in the uptake of glucose by the cell. Although conflicting reports exist, the majority view is that the increase in uptake is due to an increase in the $V_{\max}$ for glucose transport. Occasional reports of a reduction in the K_m of the glucose uptake^{2,3} have been difficult to reproduce^{4,5}. There has been little work, however, and no formal kinetic study, on the relationship between glucose uptake and tumorigenicity.

Recent observations made in this laboratory have rekindled our interest in this problem. Bramwell and Harris⁶ have shown that malignancy in hybrid cells is systematically linked to a structural alteration in the carbohydrate moiety of one particular membrane glycoprotein. Subsequent experiments provided strong circumstantial evidence that this protein had some role in glucose transport^{7,8}. Gingrich *et al.*⁹ have shown that a monoclonal antibody raised against partially purified preparations of the protein described by Bramwell and Harris delineates an antigen that behaves in a range of physiological tests as if it were the glucose transport protein. It was therefore of interest to see whether the structural change described by Bramwell and Harris was associated with any systematic functional alteration in hexose transport. Our approach has again been to compare hybrid cells in which malignancy is initially suppressed with malignant segregants derived from these hybrids. Isogenic matched pairs of this kind provide a sensitive screen for markers linked to malignancy¹⁰⁻¹⁴. We have also examined some non-malignant variants derived from tumour cells by selection for resistance to the lectin wheat-germ agglutinin⁶.

Hexose transport measurement

Hexose transport was measured by the uptake of 2-deoxy[³H]glucose (1–70 mCi mmol⁻¹, Amersham) in the following conditions. The cells were inoculated into 16-mm diameter tissue culture wells 24 h before assay. Each well received 2 ml of Eagle's minimum essential medium supplemented with 5% fetal calf serum and 5% newborn calf serum in which the cells were suspended at a concentration of $5-15 \times 10^4$ per ml. The adherent monolayers were washed twice in phosphate-buffered saline PBS and 500 μ l of the radioactive sugar in PBS were added at 20 °C. Samples were collected at appropriate times by dissolving the cell monolayer, after two washes in PBS, in 440 μ l of 0.4 M NaOH. The extract was neutralized with 10 μ l of glacial acetic acid, mixed with 10 ml of Unisolve 1 and counted in a Packard scintillation counter.

The rate of uptake was derived from a six-point time course and was measured at four to six concentrations of 2-deoxyglucose in the range 0.1–5 mM. The uptake was linear for at least 10 min. Nonspecific uptake of the radiolabel was estimated by measuring the retention of L-[³H]glucose in parallel conditions, added at a concentration of 0.1 mM at the same specific activity per ml as the 2-deoxy[³H]glucose. The mean of the measurements obtained on each of six wells was subtracted from the 2-deoxy[³H]glucose uptake measurements, and the mean number of cells per well estimated by counting the trypsinized contents of six wells in a Coulter counter. For cells in suspension, uptake of the labelled 2-deoxyglucose was measured in 1.5-ml microfuge tubes. The cells were first pelleted by centrifugation for 4 s in a Beckman microfuge, washed once with PBS and then resuspended with the radioactive sugar. Cells were collected by centrifugation followed rapidly by a wash with ice-cold PBS. Allowance was again made for nonspecific retention of the radiolabel.

At each substrate concentration, S_i , an estimate of the reaction velocity, V_i , and the variance of that estimate were calculated by linear regression through the origin of the linear portion of the plot of 2-deoxyglucose uptake against time. The K_m and $V_{\max}$ values and their standard errors were calculated by a weighted linear regression of S/V against V , essentially as described by Cornish-Bowden¹⁵. The final weight applied to

each S_i/V_i was $[V_i^2/V_{\max} V_i] [V_{\max}^2/(K_m + S_i)^2]$. Inspection of the residuals in V confirmed that these weightings were acceptable¹⁶.

For some cell lines uptake of D-glucose was measured by the silicone oil filtration centrifugation technique of Werdan *et al.*¹⁷. The kinetic constants thus obtained agreed well with those obtained by the uptake of 2-deoxyglucose as described above.

Kinetic parameters of hexose uptake in parental malignant and normal cells

Table 1 gives the K_m and $V_{\max}$ values for the parental tumour cells and the diploid cells with which they were fused. The $V_{\max}$ values are highly variable, as expected, for it is known that in some cells the $V_{\max}$ of the hexose uptake is very sensitive to cell density and other conditions of culture. The $V_{\max}$ values for some of the tumour cells are substantially lower than those for the diploid fibroblasts. There is clearly no correlation between tumorigenicity and a high $V_{\max}$ *in vitro*. On the other hand, the tumour cells and the diploid cells differ consistently in the K_m , the K_m values for the tumour cells being about half those for the diploid cells. The variation in K_m in the different fibroblast populations probably represents genotypic variation, as the duplicate values were derived from measurements on cells freshly isolated from two different animals. The PG19 tumour is derived from a C57BL mouse and is syngeneic with the C57BL fibroblasts.

The question of whether the reduced K_m seen in the tumour cells is fortuitous or whether it is closely linked to the genesis of the malignant state can now be explored by comparing the matched pairs of hybrids, as we have described.

Lymphoma $\times$ fibroblast crosses

The origin and characteristics of clone 1G, a hybrid clone derived from the lymphoma $\times$ fibroblast cross (YACIR $\times$ CBAT6T6 fibroblast), have been described previously¹⁸. The clone initially produced no tumours even with inocula of 4×10^6 cells per mouse (sublethally irradiated, syngeneic, newborn animals), but after several weeks' cultivation *in vitro*, a malignant subpopulation was generated that overgrew the cultures. Early passages of clone 1G were recloned and the secondary

Table 1 Kinetic constants of hexose uptake in malignant and normal cells

Cell type	Cell density (cells cm ⁻²)	Passage no.	K_m (mM)	$V_{\max}$ (nmol per 10 ⁶ cells per h)
PG19	2.5×10^5		0.608 ± 0.114	96.1 ± 7.8
	1.2×10^5		0.992 ± 0.119	108.2 ± 7.8
YACIR	Suspension culture		1.101 ± 0.033	44.7 ± 0.8
A9HT	2.1×10^5		1.170 ± 0.079	131.4 ± 6.3
SEWA	Suspension culture		0.683 ± 0.008	494.7 ± 2.6
TA3HaB	2.0×10^5		0.762 ± 0.078	291.2 ± 16.7
C57BL fibroblasts	4.6×10^4	2	2.510 ± 0.232	184.0 ± 12.6
	4.8×10^4	5	2.500 ± 0.494	295.7 ± 44.7
CBAT6T6 fibroblasts	3.5×10^4	3	1.622 ± 0.182	255.2 ± 16.1
	9.9×10^4	4	1.773 ± 0.153	287.9 ± 13.2
T13HT13H fibroblasts	1.1×10^5	4	1.610 ± 0.388	162.8 ± 19.3
	7.1×10^4	4	1.730 ± 0.127	387.5 ± 15.9
Rb7BnR/Rb7BnR fibroblasts	9.4×10^4	2	2.153 ± 0.155	184.6 ± 8.6
C57BL lymphocytes	Suspension culture		1.740 ± 0.085	4.5 ± 0.17

PG19 is an HGPRT⁻ derivative of a melanoma that arose spontaneously in a C57BL mouse. YACIR is a Moloney virus-induced lymphoma; an HGPRT⁻ derivative was used. A9HT is a malignant derivative of the A9 cell line selected by passage through the animal. SEWA is a polyoma virus-induced osteosarcoma. TA3HaB is an HGPRT⁻ derivative of the TA3 Hauschka cell line (details of these tumours are given in refs 10, 19). The fibroblasts were secondary cultures derived from trypsinized mouse embryos and were assayed at early passages as shown. The lymphocytes were teased from adult mouse spleen and purified by centrifugation in Ficoll-Paque.

Table 2 Kinetic constants of hexose uptake in malignant and non-malignant lymphoma × fibroblast hybrids

Hybrid	Tumorigenicity	Cell density (cells cm ⁻²)	K_m (mM)	V_{max} (nmol per 10 ⁶ cells per h)
Cl1G8	—	5.7 × 10 ⁴	3.214 ± 0.255	140.7 ± 8.9
Cl1G1	+	Nonlinear reciprocal plot		
Cl1G1T1	+	4.7 × 10 ⁴	0.725 ± 0.098	106.1 ± 7.6
Cl1G1T2	+	5.5 × 10 ⁴	1.088 ± 0.336	86.9 ± 13.9
Cl1G1T3	+	8.9 × 10 ⁴	1.063 ± 0.157	242.6 ± 21.9
Cl1G1A		Nonlinear reciprocal plot		
Cl1G1E		1.6 × 10 ⁵	1.461 ± 0.208	37.7 ± 3.2
Cl1G1F		8.9 × 10 ⁴	1.198 ± 0.165	63.1 ± 4.8
Cl1G1H		8.8 × 10 ⁴	0.666 ± 0.107	41.6 ± 3.6
Cl1G1J		9.3 × 10 ⁴	0.938 ± 0.198	57.6 ± 6.5
Cl1G8a	+	7.5 × 10 ⁴	0.851 ± 0.068	74.8 ± 2.9
Cl1G8b	+	1.5 × 10 ⁵	1.413 ± 0.263	62.8 ± 8.5
Cl1G8c	+	6.0 × 10 ⁴	0.835 ± 0.047	146.1 ± 4.2
Cl1G8bT1	+	5.2 × 10 ⁴	1.281 ± 0.168	135.9 ± 6.8
Cl1G8bT2	+	6.2 × 10 ⁴	1.349 ± 0.110	179.5 ± 8.8

clones tested for tumorigenicity. Two of these secondary clones, 1G1 and 1G8, were selected for further study. 1G8 produced no tumours with inocula of 5×10^5 cells per mouse while 1G1 produced tumours in about 90% of the animals with this inoculum.

Three tumours derived from 1G1 were explanted and the cell populations assayed *in vitro* (clone 1G1T1, T2, T3). The kinetic constants for clone 1G8 are shown in Table 2. Clone 1G1 gave markedly nonlinear reciprocal plots, but the plots for the tumours derived from 1G1 were linear and their kinetic constants are also shown in Table 2. It is clear that the K_m for hexose transport of the three 1G1 tumours is substantially lower than that of the non-malignant clone 1G8 (a factor of almost 3).

It seemed reasonable to assume that the curvilinearity of the reciprocal plots for clone 1G1 was due to a mixture of cells in the population assayed. Clone 1G1 was therefore re-cloned to give tertiary clones 1G1A, E, F, H and J. Clone 1G1A still gave a curvilinear plot, but clones E, F, H and J gave linear plots with the K_m and V_{max} values shown in Table 2. Each of these clones gave the low K_m characteristic of tumorigenic cells. These results thus support the view that tumorigenic variants are generated in the initially non-malignant cell populations and progressively overgrow the cultures. As no tumours were produced by direct injection of clone 1G8, we attempted to select from this clone subclones capable of growth in agarose, in the hope that such subclones might be enriched for cells capable of progressive growth *in vivo*. From three dishes, each seeded with 10^5 cells in agarose as described by Steinberg and Pollack¹⁹, five primary colonies were obtained, of which three survived isolation and further subculture (clones 1G8a, b, c). These gave 80–100% take incidences at 5×10^5 cells per mouse. Table 2 shows the K_m and V_{max} values for these clones and for tumours derived from them (1G8bT1 and T2). Both the clones themselves and the tumours produced by them have K_m values two to three times

lower than that of the non-tumorigenic clone 1G8 from which they were derived.

The association between reduced K_m for hexose transport and tumorigenicity is thus not fortuitous. Malignant derivatives obtained directly by the inoculation of the hybrid cell population into the animal or indirectly by selection in semi-solid medium systematically show a reduction in K_m compared with the non-tumorigenic hybrids from which they were derived.

Melanoma × fibroblast crosses

Crosses between the PG19 melanoma derivative and diploid fibroblasts homozygous for the T13H translocation produced only 4 tumours out of a total of 167 mice given inocula of $2-3 \times 10^6$ cells per animal¹⁸. The kinetic constants for two clonal populations of this cross, clones 7 and 8, and a tumour produced from clone 8 (clone 8T1) are shown in Table 3. The hybrids in which malignancy is suppressed show high K_m values (more than three times the value for the parental PG19 tumour cells); the tumour produced by clone 8 shows a marked reduction in K_m compared with that of clone 8 itself.

A melanoma derivative selected for resistance to wheat-germ agglutinin

Bramwell and Harris⁶ found that selection of PG19 cells for their ability to grow in lethal concentrations of wheat-germ agglutinin produced derivatives with greatly reduced tumorigenicity. One such line (C) produced no tumours in 17 animals with inocula of 5×10^4 cells per animal. This line was re-cloned and one of the secondary clones, PG19WGAR clone C2, was assayed. This non-tumorigenic clone had a high K_m comparable with that of the PG19 × T13HT13H fibroblast hybrids in which malignancy was suppressed (Table 3). Changes in tumorigenicity produced by wheat-germ agglutinin selection are thus also associated with changes in the K_m for hexose transport.

Fibrosarcoma × lymphocyte crosses

Crosses between diploid lymphocytes and the malignant L-cell derivative A9HT show suppression of malignancy²⁰. Assay of two highly suppressed clones, clones 3 and 4, showed their K_m values to be 2.240 ± 0.160 and 2.080 ± 0.120 (Table 4), compared with 1.170 ± 0.079 for the parental tumour cell (Table 1). A tumour derived from another such clone (A9HT × C57BL lymphocyte clone 2T1) resembled the parental tumour cell in having a K_m value of 1.123 ± 0.046 .

Fibrosarcoma derivatives selected for resistance to wheat-germ agglutinin

Selection of A9HT cells for resistance to wheat-germ agglutinin produced some derivatives that had lost tumorigenicity and others that retained it²¹. Clones A9HTWC and A9HTWD were both resistant to $25 \mu\text{g ml}^{-1}$ wheat-germ agglutinin, but whereas clone WC failed to produce any tumours with inocula up to 6.6×10^6 cells per animal, clone WD remained as tumorigenic as the original A9HT parent cell. Table 4 shows that the K_m for hexose transport of the non-tumorigenic clone is more than twice that of the tumorigenic clone.

Table 3 Kinetic constants of hexose uptake in melanoma × fibroblast hybrids and in a lectin-resistant melanoma derivative

Cell type	Tumorigenicity	Cell density (cells cm ⁻²)	K_m (mM)	V_{max} (nmol per 10 ⁶ cells per h)
PG19 × T13HT13H Clone 7	—	2.1 × 10 ⁵	2.348 ± 0.339	195.0 ± 16.4
PG19 × T13HT13H Clone 8	—	5.3 × 10 ⁴	3.587 ± 0.572	160.5 ± 18.4
		7.9 × 10 ⁴	2.533 ± 0.466	132.5 ± 16.2
PG19 × T13HT13H Clone 8T1	+	2.1 × 10 ⁵	1.480 ± 0.051	204.3 ± 3.8
PG19WGAR Clone C2	—	2.0 × 10 ⁵	2.40 ± 0.230	173.5 ± 11.5

Table 4 Kinetic constants of hexose uptake in fibrosarcoma × lymphocyte hybrids and in lectin-resistant fibrosarcoma derivatives

Cell type	Tumorigenicity	Cell density (cells cm ⁻²)	K_m (mM)	V_{max} (nmol per 10 ⁶ cells per h)
A9HT × C57BL lymphocyte C13	—	2.6 × 10 ⁵	2.240 ± 0.160	247.6 ± 13.2
A9HT × C57BL lymphocyte C14	—	2.5 × 10 ⁵	2.080 ± 0.120	451.3 ± 20.8
A9HT × C57BL lymphocyte C12T1	+	2.0 × 10 ⁵	1.123 ± 0.046	119.4 ± 2.8
A9HT WC	—	1.8 × 10 ⁵	1.685 ± 0.151	133.9 ± 7.6
A9HT WD	+	1.8 × 10 ⁵	0.731 ± 0.087	120.4 ± 7.9

Table 5 Effect of continuous culture *in vitro* on the kinetic parameters of hexose uptake in osteosarcoma × fibroblast hybrids

Hybrid clone	No. of days in continuous culture	Cell density (cells cm ⁻²)	K_m (mM)	V_{max} (nmol per 10 ⁶ cells per h)
C1	3	2.1×10^5	2.986 ± 0.140	445.3 ± 16.3
	7	1.4×10^5	2.499 ± 0.267	243.6 ± 19.1
	9	1.6×10^5	1.896 ± 0.130	354.6 ± 17.2
	16	2.0×10^5	1.156 ± 0.108	129.5 ± 9.42
	22	2.9×10^5	1.420 ± 0.376	462.6 ± 39.0
F	6	1.8×10^5	3.218 ± 0.529	372.1 ± 37.6
	13	2.4×10^5	2.220 ± 0.052	723.0 ± 11.0
	40	6.3×10^4	1.363 ± 0.092	240.6 ± 7.4

Polyoma virus-induced osteosarcoma × fibroblast crosses

As previously described, hybrids between SEWA and diploid cells are highly unstable^{18,22}. Malignant segregants are generated in this cross at a very high frequency so that the tumorigenicity of the hybrid clones is variable. Hybrids that initially fail to produce tumours become tumorigenic again on continued cultivation *in vitro*. Early assay of a fresh set of nine hybrid clones isolated from a cross between SEWA and fibroblasts homozygous for the Rb7BnR translocation showed K_m values for these clones ranging from 0.8 to 2.9 mM; in some cases the reciprocal plots were initially curvilinear, as described for the YACIR × fibroblast crosses. The clones isolated were obviously a heterogeneous collection. Two clones, C1 and F, which initially gave high K_m values, were grown continuously *in vitro* and their K_m values determined at intervals. The results (Table 5) show that on continued cultivation the K_m values fell progressively. Clone F, tested *in vivo*, initially produced no tumours, but soon became tumorigenic. The tumours produced from clones C1 and F gave lower K_m values than those of the

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cells inoculated. There is clearly further selection for lower K_m *in vivo*.

Discussion

Our findings leave little doubt that malignancy, as defined by the ability of cells to grow progressively *in vivo*, is closely linked to a decrease in the K_m of the membrane hexose transport system. As the difference between malignant and non-malignant cells becomes more pronounced at lower external hexose concentrations, it is not difficult to see how the reduction in K_m might confer a selective advantage on malignant cells *in vivo*. The cell density in a primary tumour nodule is high and the blood supply precarious, so that the availability of hexose could easily be limiting. A decrease in the K_m of the hexose transport system might thus make the malignant cell a more effective scavenger of whatever hexose is available than the normal cells with which it must compete.

Two general questions arise concerning the mechanism of the change in K_m . The first is whether it affects only the hexose transport system or whether other membrane transport systems behave similarly in malignant cells. The second question is whether the change in K_m reflects the operation of a structurally modified or different hexose transport system, or whether this change is produced as a secondary consequence of more generalized membrane changes. The answer to the latter question must wait until the elements of the hexose transport system have been unequivocally identified and subjected to formal structural analysis. In the light of our previous findings⁶, we are exploring the possibility that modification of the carbohydrate moiety of the hexose transport protein influences the K_m of the transport process.

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LETTERS TO NATURE

Innermost parts of accretion disks are thermally and secularly stable

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There is strong evidence that various types of exotic astronomical objects (quasars, active galactic nuclei, Cyg X-1, SS433) can be explained by accretion disks orbiting black holes. Most of the proposed models of such objects are plagued by instabilities. It is widely held, for example, that all accretion disks must be thermally and secularly unstable in their innermost parts, which seems to be in direct conflict with observations. Much effort has been made to find a stabilizing mechanism operating in the innermost parts of the disks. Here I show that such a mechanism does exist. It is of general relativistic origin, is purely mechanical—operating independently of viscosity and other micro-physical processes—and is similar to the mass loss caused by Roche lobe overflow in close binaries.

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It is generally believed that the inner regions of standard, thin accretion disks are both thermally and secularly ($L-E$) unstable. This is because the most important stability condition,

$$\left. \frac{d \ln Q^+}{d \ln H} \right|_U < \left. \frac{d \ln Q^-}{d \ln H} \right|_U \quad (1)$$

is not satisfied by any one of the possible relevant cooling mechanisms (Q^-) when the dissipation, the rate of heat generation (Q^+), is determined by the standard viscosity law. In equation (1), H denotes the height of the disk and U the surface density.

Piran¹ gives the most general discussion of the stability problems in terms of four quantities k, l, m, n

$$k \equiv \left. \frac{d \ln Q^-}{d \ln H} \right|_U, \quad l \equiv \left. \frac{d \ln Q^-}{d \ln U} \right|_H \quad (2)$$

$$m \equiv \left. \frac{d \ln \nu}{d \ln H} \right|_U, \quad n \equiv \left. \frac{d \ln \nu}{d \ln U} \right|_H \quad (3)$$

which describe phenomenologically a wide range of different viscosity laws and cooling mechanisms, in particular all those discussed in the accretion disk context. In equation (3), the quantity ν is the kinematic viscosity. The standard viscosity

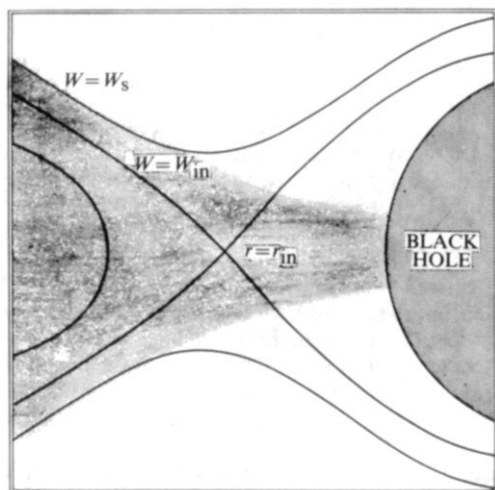


Fig. 1 Equipotential surfaces close to the inner edge of the disk.

law has $m = 2$, $n = 0$. Non-standard viscosity laws have $m < 2$, $n = 0$.

A recently discovered² mass loss mechanism which operates close to the inner edges of accretion disks orbiting black holes removes both thermal and secular instabilities. There are no instabilities even in the extreme case when the ratio of radiation pressure to the total pressure, $\beta = 1$, and when the Shakura-Sunyaev α -viscosity parameter, $\alpha = 1$. The conclusion holds for both standard and non-standard viscosity laws.

Piran's arguments and his stability conditions are used here. After concluding that there is no combination of any known cooling mechanism with the standard viscosity law which gives rise to a stable inner disk region (radiation pressure and electron scattering-dominated), Piran remarks that a secondary wind escaping from the disk surface would have a stabilizing effect. He admits, however, that generally this wind would be too small to change the main conclusion significantly. Nevertheless, he gives explicit forms of the necessary and sufficient stability conditions for thermal and secular stability of a disk with a secondary wind. The conditions are general and valid not only for winds, but for any mass loss from the surface. The mass loss is expressed by

$$\dot{M} \sim U^* H^* \quad (4)$$

and the stability conditions yield

$$(8s - 3r) + (51s - 9r)\beta - (3s + 12r)\beta^2 - X^2(m - k) > 0 \quad (5a)$$

$$r(n + 1 - l) - s(m - k) > 0 \quad (5b)$$

$$[2m + 8(n + 1)] + [9m + 51(n + 1)]\beta - 3[m + n + 1]\beta^2 > 0 \quad (5c)$$

$$[sm - (n + 1)r] - Y^2[ml - (n + 1)k] > 0 \quad (5d)$$

The explicit form of X^2 and Y^2 is not relevant here.

General relativistic effects in the gravitational field of a black hole cause a characteristic behaviour of the equipotential surfaces close to the inner edge of an accretion disk: one of the equipotentials, $W = W_{in}$, crosses itself on the edge (see Fig. 1). If the surface of the disk, $W \approx W_s$, slightly overflows the critical equipotential $W = W_{in}$, then mechanical equilibrium is slightly destroyed, causing mass loss. The physical picture is analogous to the case of Roche lobe overflow in close binaries.

Abramowicz, Calvani and Nobili³ give expressions for the mass loss rate and efficiency of the cooling mechanism connected with the mass loss assuming only mechanical, not thermal, equilibrium. Their equations (5.19) and (5.13) valid close to the inner edge of the disk, that is for $r \approx r_{in}$, read

$$\dot{M} \sim UH \quad (6)$$

$$Q^- \sim UH^3 \quad (7)$$

Thus, for this particular mass loss mechanism $s = 1$, $r = 1$, $l = 1$ and $k = 3$ (for $r \approx r_{in}$).

This result, when combined with the general stability criteria of equation (5), proves that accretion disks orbiting black holes

are thermally and secularly stable close to their inner edges. This is true for both standard and non-standard viscosity laws and it does not depend on any particular value of α or β .

Note that the stabilizing mechanism is purely general relativistic in its origin. This may be the only known situation when a general relativistic effect stabilizes a configuration, especially a rotating one.

The physics beyond this formal proof is simple: consider a small, purely thermal (that is with $U = \text{constant}$) perturbation which produces an excess heat ($Q^+ > Q^-$). If the excess heat cannot be removed from the disk, the disk expands. This causes increased Roche lobe overflow: the location of the Roche lobe depends only on the angular momentum at $r = r_{in}$ and does not change during the expansion. Because the cooling increases very strongly with the increasing overflow and the heating does not, the disk reaches the state $Q^- = Q^+$ and achieves thermal stability.

More detailed treatment of this phenomenon will be published elsewhere where I shall discuss the radial size of the region, $r_{in} \leq r \leq r_*$, affected by this stabilizing mechanism. The size, of course, depends strongly on the particular situation. A rough estimate gives $r_* > H(r_{in})f(r_*) \approx \text{few } r_{in}$, where $f = (\text{thermal time scale/free-fall time scale})$. For the supermassive-black-hole case this is enough to stabilize the whole inner (radiation pressure- and electron scattering-dominated) region of the disk.

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Milliarcsecond structure of BL Lac during outburst

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The well known object BL Lacertae is the prototype of a class of compact extragalactic sources which display rapid flux and polarization changes at radio and optical wavelengths and a nearly featureless optical spectrum¹. After a period of relative quiescence, BL Lac has recently undergone a violent outburst at radio wavelengths, accompanied by rapid changes in degree of polarization and position angle (Fig. 1)^{2,3}. For a better understanding of the physical mechanism responsible for the variable emission, we have mapped the radio structure at 5 and 10.6 GHz at three epochs during the large flux outburst of 1980 with an intercontinental VLBI array using telescopes in Bonn, West Germany; Green Bank, West Virginia; Westford, Massachusetts; Fort Davis, Texas; and Owens Valley, California. The synthesized beam had a resolution of about 1.0 m arc s at λ 6 cm and 0.5 m arc s at λ 2.8 cm, corresponding to linear sizes of 5.9 and 2.9 lyr (light year) at the source (using a redshift⁴ of 0.0695 and a Hubble constant of $55 \text{ km s}^{-1} \text{ Mpc}^{-1}$). We show here that comparison of the size and flux density of the core component with the flux history provides evidence for relativistic beaming effects, independent of detailed model considerations. The flux densities shown in Fig. 1 were obtained at the University of Michigan radio observatory.

The observations were made at three epochs: 1980.41 (λ 6 cm), 1980.73 (λ 6 cm) and 1980.93 (λ 2.8 cm). During each epoch, one telescope had a technical failure so that only four telescopes were used in each mapping analysis. The telescopes used the MKII VLBI recording scheme⁵ and were equipped

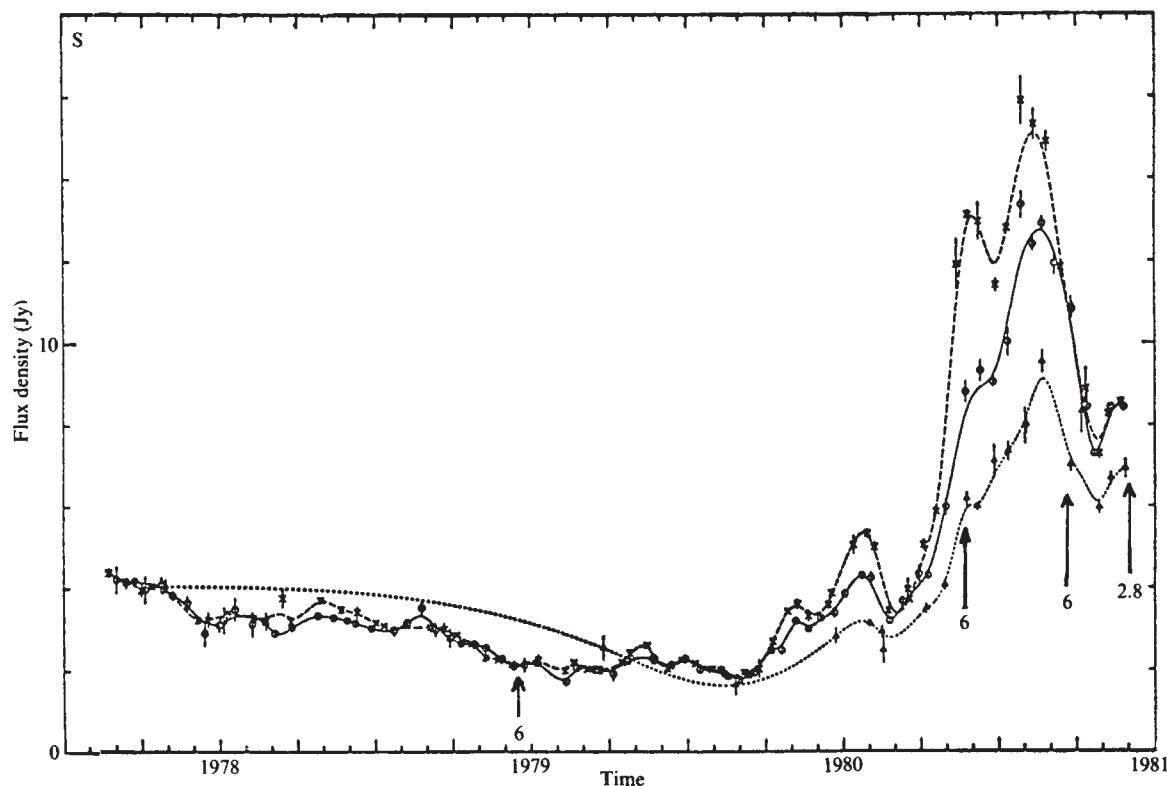


Fig. 1 Flux density compared with time at 4.8 GHz ($\triangle \cdots \triangle \cdots$), 8.0 GHz ($\bullet \cdots \bullet \cdots$) and 14.5 GHz ($\times \cdots \times \cdots$). Data points are 2-week averages. Epochs of VLBI observations are shown by arrows where the number indicates wavelength of each observations.

with maser time standards. All observations were made using left circular polarization and a receiving bandwidth of 2 MHz. The data were correlated using the Caltech/JPL VLBI correlator. The mapping was done at the University of Iowa using a hybrid algorithm⁶ utilizing closure phase. For all three epochs, the resulting fits to the data were very good, and the maps are thought to be reliable to the 5% contour level. An example of the visibility data and corresponding hybrid model fit is given in Fig. 2 for the 1980.93 data at λ 2.8-cm wavelength.

The λ 2.8-cm hybrid map is shown in Fig. 3a, while the two λ 6-cm maps are shown in Fig. 4a, b. In each case, the restoring beam has been chosen by fitting an elliptical gaussian to the main beam of the array power pattern, which is obtained by Fourier transforming the transfer function for each observation. A remarkable aspect of the structure at all three epochs which is not apparent on the hybrid maps is the extreme narrowness of the source. For both λ 6-cm observations, the source was completely unresolved when the baselines were aligned approximately east-west. The λ 2.8-cm data also show this effect, although there is some east-west resolution on the transatlantic baselines. This dominant north-south structure has been seen with more limited VLBI measurements⁷⁻⁹ as early as 1971 and apparently indicates that there is an alignment mechanism in the central source which is stable compared with the time scale of individual flux outbursts.

To clarify the detailed north-south structure, we have shown one-dimensional profiles of brightness temperature along a position angle of 0° for each map. Figure 2b shows the λ 2.8-cm profile, while Fig. 5 shows the λ 6-cm profiles at both epochs. In addition, we have shown the north-south brightness profile for the three-component gaussian model of Pearson and Readhead¹⁰ based on data taken at λ 6-cm in 1978 December when BL Lac was dormant (see Fig. 1). To compare brightness temperatures in a uniform way, we have used the same restoring beam for all three λ 6-cm profiles. This beam is too small for the December 1978 model (because only US baselines were used) so that the detailed structure of the components is probably unreliable. We have also convolved our data with the same restoring beam of Pearson and Readhead and find no evidence

of the southerly component extending ~ 10 mas (60 μ as) seen in their 1978 map.

Comparison of the λ 2.8 map (Fig. 3) with the flux history data of Fig. 1 shows that the source is much larger than would be expected from simple light travel time arguments. The current outburst can be dated from ~ 1979.8 when the total flux density is ~ 2 Jy at λ 6 cm and λ 2.8 cm. Without relativistic effects, the maximum source size in ~ 1980.9 would be 1.1 ly, whereas the north-south extent of the λ 2.8-cm map is ~ 4.5 ly. This discrepancy could be understood in several ways: (1) simultaneous, independent brightening of causally unconnected regions; (2) activation by a single 'trigger' mechanism located approximately midway between two distant emitters; or (3) relativistic beaming effects causing a time 'speed-up' in the observed flux changes. Explanation (1) seems very improbable, particularly considering the 3-yr dormant period which preceded the outburst, while explanation (2) requires a highly specialized geometry.

The relativistic beam model¹¹ has been widely used to explain the properties of compact radio sources¹²⁻¹⁴. The model consists of a central object with two opposed particle beams moving with relativistic bulk velocities. If the line of sight to the observer is nearly parallel to the beam axis, he will see the forward beam Doppler-boosted in intensity. The true angular source size is the apparent size as observed in the VLBI data, but the flux variations are observed 'speeded up' by the Lorentz factor, which in the present case is $\gamma \sim 4$.

A further argument supporting relativistic beaming in this source is the anomalously high brightness temperatures implied by the flux density variations. In the absence of relativistic effects, the inferred brightness temperature of the core component would be at least 10^{13} K based on the light travel size. This violates the inverse Compton limit of $T_B \leq 10^{12}$ K for an opaque synchrotron emitter. On the other hand, the observed brightness temperatures (based on the actual measured angular sizes) are $T_B \leq 3 \times 10^{11}$ K for all three observations.

The λ 6-cm maps do not have sufficient angular resolution to show the details of the evolution of the jet during the outburst. However, comparison of the 1980.41 and 1980.73 brightness

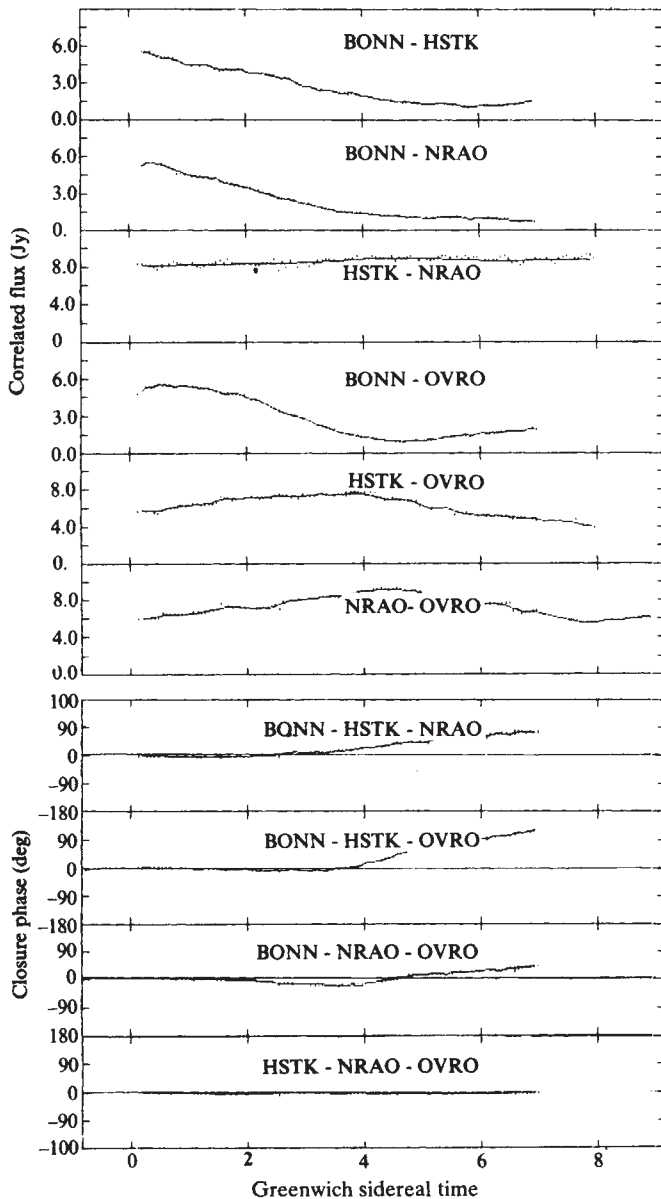


Fig. 2 Four-min averages of fringe amplitude and closure phase for the 1980.93 data at 10.6 GHz. Also shown is the fit from the hybrid map shown in Fig. 3a.

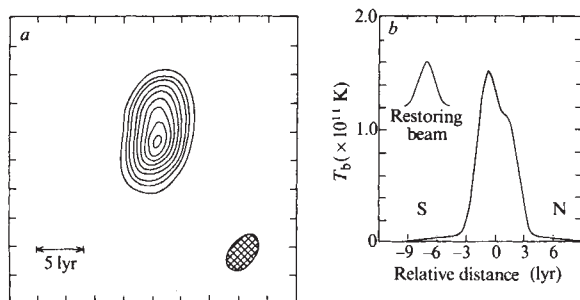


Fig. 3 a, Hybrid map of BL Lac at 10.6 GHz, epoch 1980.93. Contours are 5, 15, 25, 35, 45, 55, 65, 75, 85 and 95% of peak flux. b, One-dimensional profile of peak brightness temperature along position angle 0° in map in a.

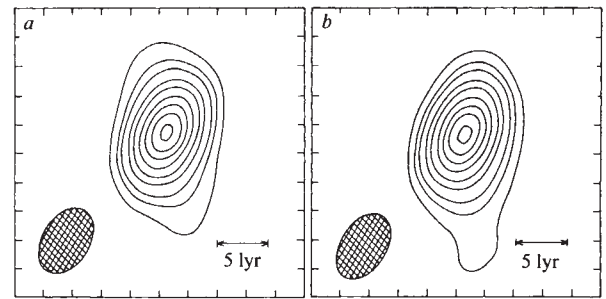


Fig. 4 a, Hybrid map of BL Lac at 5 GHz, epoch 1980.41. Contours as in Fig. 3a. b, Epoch 1980.73, same as in a.

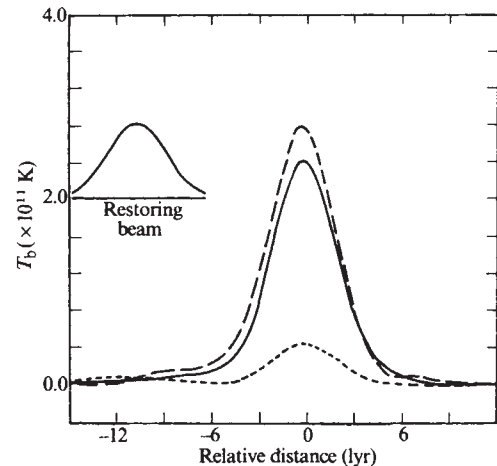


Fig. 5 One dimensional profiles of peak brightness temperature along position angle 0° for 5 GHz maps at three epochs. Dotted line is epoch 1978.93, solid line is epoch 1980.41 and dashed line is epoch 1980.73. Note that the relative position between epochs can not be determined from the VLBI data and is not necessarily as shown here. Also the same restoring beam has been used for all three epochs. This over-resolves the 1978.93 data (see text).

profiles (Fig. 5) show that there has been a small, but definite increase in the core size, of ~ 0.5 ly. This is actually a lower limit, as the true size has been convolved with a restoring beam whose dimensions are comparable with the core size. In addition, all three profiles show a weak, extended southerly component. The detailed nature of this component is uncertain because of the limited dynamic range of the maps. However, we have found differences in the closure phase on identical baseline triangles in the comparison of 1980.41 and 1980.72 data which we are confident are caused by changes in the extended southerly structure. The extended structure is not seen on the λ 2.8-cm profile, indicating that perhaps the extended emission has become optically thin between λ 6 and λ 2.8 cm.

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Diffuse interstellar absorption bands between 2.9 and 4.0 μm

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IRS7, the obscured M supergiant near the galactic centre, is an excellent source against which to study interstellar absorption bands near 3- μm wavelength. Previously we considered¹ the 3.4- μm absorption band as evidence that interstellar grains include an organic component. We report here superior data between 2.9 and 4.0 μm , and find a broad absorption trough with numerous discrete features stretching from 2.9 to 3.6 μm . Although no definite identification of the material producing this absorption is possible, there is some indication that complex organic molecules may be involved.

Because of the strong and variable telluric absorption features in the waveband of interest, we now describe our observing methods in some detail. We used the IR photometer-spectrometer at the $f/15$ focus of the 3.9-m Anglo-Australian Telescope. In this instrument a circular variable filter (CVF) is scanned rapidly and repeatedly across the image of a source; in this case each spectral scan between 2.9 and 4.0 μm required 5.8 s. The resolution of the CVF is $\lambda/\Delta\lambda \sim 100$. Scans were alternated in direction, and at the end of each pair of scans the telescope was driven back and forth between IRS7 and a patch of sky 80 arc s to the north. This procedure ensured that the data were not contaminated by signals from other sources in the galactic centre area appearing in the reference sky beam. A circular aperture of 3.5 arc s diameter was used to reject nearby sources, in particular the very cool object IRS3. The resulting spectrum shows a weaker contribution from cool dust at 4 μm than any previously published. Indeed, IRS7 may have no intrinsic excess radiation from circumstellar dust, and the contribution we see may be contamination from nearby sources.

Approximately every 5 min we made observations in exactly the same manner of the nearby K0 giant star γ Sgr. Five separate observations of IRS7 were individually bracketed by observations of γ Sgr. All observations were made when both objects lay within 10° of the zenith. The observations of γ Sgr monitored changes in the telluric absorption bands. The changes found on this occasion were quite small.

Figure 1 shows our raw data in the vicinity of the strongest telluric features, between 2.9 and 3.45 μm . Figure 1b refers to the ratio IRS7/ γ Sgr, and shows the five separate observations, displaced for clarity, and their mean: the major features repeat well from one spectrum to the next. Note the narrow absorptions near the channels 23 and 30. We present the raw spectra of γ Sgr, showing the telluric absorptions in Fig. 1a. These are displaced from the features in the quotient spectra, indicating that the latter do not arise from poor cancellation of the telluric features.

An independent observation made in July 1980 enables a final check of these features. This observation was less favourable because IRS7 and γ Sgr lay at a greater zenith distance and a different CVF gave a resolution of only 50. Finally, the 1980 data used a larger aperture and smaller beam separation. Nonetheless, between 2.9 and 3.45 μm the same spectral features were seen except for the first few data points at the short-wavelength end of the spectrum.

Also shown in Fig. 1a is the ratio of the spectrum of γ Sgr to a G dwarf star, whose spectrum is expected to be featureless in this spectral region. The telluric cancellation is not as good on this occasion because of the greater time gap between the two observations. This ratio indicates that the spectral features

observed in IRS7 are not introduced by the use of γ Sgr as a calibrator.

Spectra obtained of the M supergiant VX Sgr confirm that it does not show any absorption features in the 2.9–4.0 μm region. Thus all the absorption bands in the spectrum of IRS7 are of interstellar origin.

Figure 2 shows the resultant spectrum, including the portion from 2.0 to 2.5 μm . The deep photospheric CO absorption of the M supergiant is clearly seen in this spectral region, and as expected there is no indication of the 1.9 or 2.7 μm steam absorption bands. Outside the CO band we expect IRS7 to show a black-body continuum. Note that there is roughly a 10% uncertainty in the relative heights of the 2 and 3 μm spectra caused by the necessary use of a small aperture and the presence of seeing of 1–2 arc s. The broken line on Fig. 2 indicates where the 1980 spectrum digresses from the 1981 data.

To reveal the depth of the absorption we have constructed a continuum in Fig. 2 which we have forced to fit through the 2.0–2.25 and 3.6–3.95 μm sections of the data. Three parameters describe such a continuum: the black-body temperature of the star, assumed to be 3,200 K, the extinction at a chosen wavelength (2.2 μm : 2.5, derived from ref. 1), and the form of the reddening law. For the latter we use $A_\lambda \propto \lambda^{-n}$, where A_λ is the reddening in stellar magnitudes at wavelength λ . We assumed $n = 2$ based on ref. 3. With any reasonable adjustment of these parameters we cannot fit the designated continuum points. To do so we add a cool black-body component representing emission from dust. This may arise in IRS7 itself, in the nearby IRS3, or in fainter, unmapped sources. Two more parameters are then involved: the dust temperature and the intensity of the dust emission. The continuum plotted in Fig. 2 has a dust component of 550 K having 0.19 of the star's flux at 3.6 μm .

Relative to this continuum we find a complex of absorption

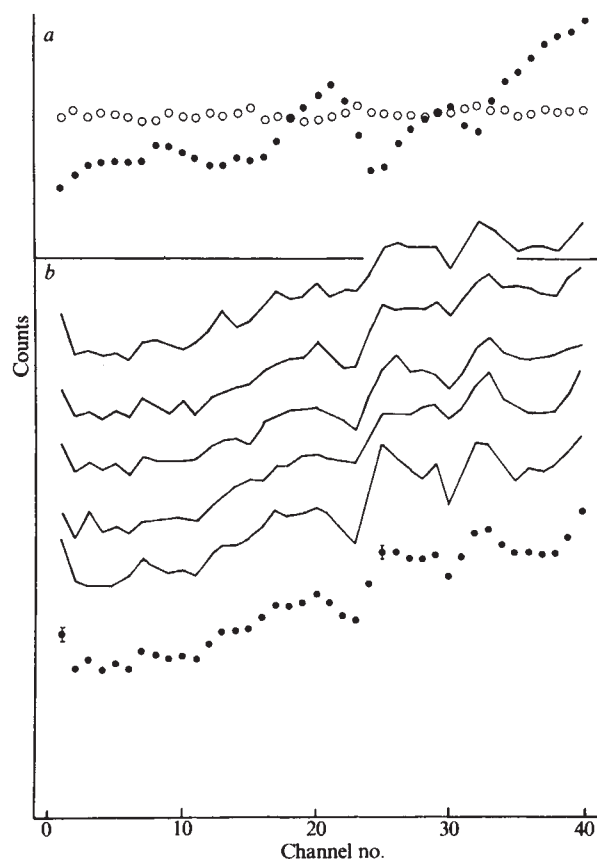


Fig. 1 b, IRS7 divided by the standard γ Sgr plotted against channel number in the region of the telluric bands (2.9–3.45 μm). The solid curves are based on five individual sets of observations obtained as described in the text. ●, The mean spectrum. a, The raw spectrum of γ Sgr showing the telluric bands (●) and the ratio of γ Sgr (K0) to that of a G dwarf star (○).

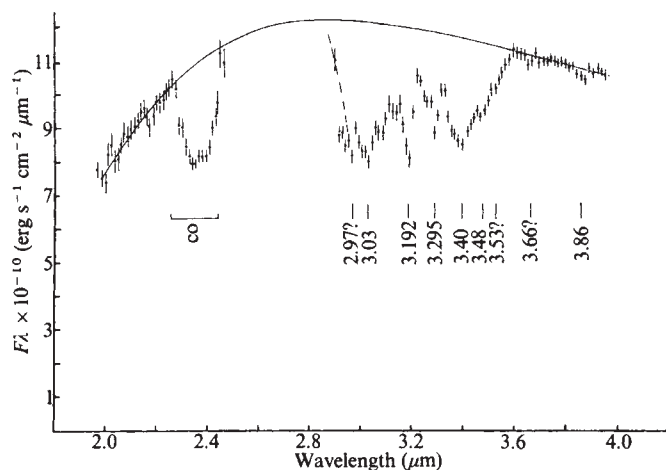


Fig. 2 The 2.0–4.0 μm spectrum of IRS7. The solid curve is the estimated position of the continuum (see text). The dashed curve is the July 1980 spectrum where it deviates from the May 1981 spectrum.

bands stretching from 2.9 to 3.6 μm . Two dominant bands lie at 3.03 and 3.40 μm , and have optical depths of 0.41 and 0.32 respectively. The increased optical depth of the 3.4 μm feature relative to our previous result¹ reflects the improved definition of the continuum. This optical depth would be further increased if the redness between 3.6 and 3.95 μm were due to absorption rather than to the addition of cool dust.

Figure 2 shows all of the absorption features we believe to be real, and a few (marked?) which are less certain. The absorption clearly breaks up into many discrete features and two of these (at 3.192 and 3.295 μm) are quite narrow. Whilst the broad features centred at 3.03 μm and 3.40 μm presumably arise in solid grains, the narrow features may have a gaseous origin. We have found no convincing identification for either, although many hydrocarbons have stretching absorptions in this spectral range. However, laboratory data are not available for even quite simple, incompletely bonded molecules (for example CH_3) such as might be found in the interstellar environment. Quite narrow absorption bands are found in the absorption spectra of some solids, such as methane⁴ and methyl alcohol⁵.

Suitable data on the absorption properties of solid materials are not available, and laboratory work is needed to match the present spectra. We see no evidence for water ice in the available data. The absorption band at 3.03 μm is displaced from that of water (3.06 μm) and is narrower. If water ice is present, it contributes little to the absorption. Similarly, solid ammonia (2.91 μm) is not present. Molecules involving carbon and hydrogen, on the other hand, can produce absorption near both 3.0 and 3.4 μm , and are thus particularly attractive identifications. Organic molecules containing OH and NH bonds also cause absorption near 3.0 μm (refs 6, 7). The wavelengths of CH, NH and OH vibrations depend critically on the composition of the solid and only a few cases⁵ of astronomical interest have been discussed in the literature. The H bonded solid complex of H_2O , CH_3OH and NH_3 (ref. 5) has a feature due to NH at 2.97 μm but there is no correspondence with the rest of the spectrum.

It has been suggested recently⁸, that surface functional groups attached to reactive sites on small carbon grains may be responsible for the IR features seen in IRS7. Although there are some interesting wavelength coincidences (aromatic —CH (3.3 μm), — CH_3 (3.4, 3.5 μm), —CHO (3.5, 3.65 μm)) no information has been given on band strengths and shapes to enable a detailed comparison.

We have looked at published spectra of specific grain models involving organic polymers which could be possibilities for the organic component of interstellar grains. The polymer-like material in carbonaceous chondrites⁹ show absorption due to CH near 3.3 μm and weak absorption at 3.0 μm . Clearly this material will not produce a fit to the astronomical data even over a restricted wavelength region. However, hydrated silicates of

meteoritic origin show a broad absorption feature centred near 3 μm ¹⁰. This may be relevant in view of the possible identifications of the 9.7 μm and 18 μm features in the galactic centre with silicates. We consider it significant that the yellow component of UV tholin^{2,9} looks qualitatively similar to that of IRS7 in the 3.4- μm region. In particular the shoulder at 3.36 μm , the central component at 3.4 μm and the component at 3.48 μm are present in the laboratory data with wavelengths agreeing to within ± 0.02 μm . This could indicate the existence of complex organic molecules in grains^{2,11,12}. However, this material does not produce significant absorption near 3 μm as required by the IRS7 data. It is interesting that the satellite features at 3.36, 3.48 μm are also seen in the spectrum of polyformaldehyde suggesting that H_2CO may be present as a structural unit¹³.

We cannot rule out the possibility that a mix of simple organics might match the observed spectrum. This requires further investigation using laboratory data. However, as our data are of relevance mainly to the properties of dust in the diffuse interstellar medium it will be more appropriate to look at refractory materials related to the UV component of tholins such as the non-volatile residue produced in the laboratory experiments designed to simulate conditions in the interstellar medium described by Greenberg¹² and with predictions from the Hoyle–Wickramasinghe model¹¹. The latter comparison has shown a remarkable similarity between the spectrum of IRS7 and that of dried bacteria (*Escherichia coli*) which will be reported elsewhere¹⁴.

Finally emission features are found in this waveband in some H II regions and carbon-rich planetary nebulae (for example, NGC7027)¹⁵. These normally comprise a strong, narrow peak at 3.29 μm and a weaker, broader feature peaking at 3.40 μm . In this respect they mimic features in the relevant portion of the absorption spectrum of IRS7. However, the 3.03 and 3.192 μm features are not seen in emission in any objects. An interpretation of the absorption spectrum of IRS7 which simultaneously predicted the more restricted range of the emission bands would be very satisfying.

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Fractal dimensions of landscapes and other environmental data

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Mandelbrot¹ has introduced the term 'fractal' specifically for temporal or spatial phenomena that are continuous but not differentiable, and that exhibit partial correlations over many scales. The term fractal strictly defined refers to a series in which the Hausdorff–Besicovitch dimension exceeds the topological dimension. A continuous series, such as a polynomial, is differentiable because it can be split up into an infinite number of absolutely smooth straight lines. A non-differentiable continuous series cannot be so resolved. Every attempt to split it up into smaller parts results in the resolution of still more structure or

Table 1 Estimated D values for various environmental series

Location	Property	Lag	D as lag $\rightarrow 0$	D at max. slope	Ref.
Wales	Soil—sodium content	15.2 m	1.7–1.9*	—	7
	—stone content	15.2 m	1.1–1.8*	—	7
	(both over four directions)				
England	Soil—thickness of cover loam	20 m	1.6*	—	7
England	Soil—electrical resistivity (4 directions)	1 m	1.4–1.6*	—	7
England	Surface of airport runway	30 cm	1.5†	—	8
Deserts in Africa and America	Soil—mean cone index	~1 km	1.9‡	—	9
	—silt + clay in 0–15 cm layer	~1 km	1.8‡	—	9
	—mean diameter of surface stones	~1 km	1.8‡	—	9
	—coarse sand fraction in 0–15 cm layer	~1 km	1.8‡	—	9
	Vegetation cover	~1 km	1.6‡	—	9
South Africa	Gold	Various	1.9*	—	2
Australia	Soil—phosphorus level	5 m	2.0‡	—	10
	—pH	5 m	1.5‡	—	10
	—potassium level	5 m	1.6‡	1.1‡	10
	—bulk density	5 m	1.5‡	—	10
	—0.1 bar water	5 m	1.5‡	—	10
France	Iron ore in rocks				
	—chlorite	15 μ m	1.6*	—	11
	—quartz	15 μ m	1.9*	—	11
	—quartz	5 cm	1.6*	—	11
	—iron	5 cm	1.5*	—	11
	—iron (E–W)	100 m	1.7*	—	11
	—iron (N–S)	100 m	1.8*	—	11
	—iron (E–W)	500 m	1.6*	—	11
	—iron (N–S)	500 m	1.9*	—	11
France	Sea anemones	10 cm	1.6§	—	12
Chad	Rainfall	1 km	1.7*	—	13
Mauritania	Iron ore	3 m	1.4*	—	2
Ivory Coast	Groundwater levels				
	Piezometer 1	1 day	1.6*	—	2
	2	1 day	1.7*	—	2
	3	1 day	1.8*	1.3*	2
	4	1 day	1.3*	1.1*	2
Canada	Oil grades	60 cm	1.7*	—	2
Chile	Copper grades	2 m	1.7*	—	2
France	Topographic heights	10 m	1.5*	1.1*	2
USA	Soil—sand content	10 m	1.6–1.8*	—	14
	—pH	10 m	2.0*	—	14
Worldwide	Crop yields	1–1,000 m	1.6–1.8‡	—	15
India	Water table depth	250 m	1.6*	—	16

* Estimated from variogram. ‡ Estimated from block variance. † Estimated from power spectrum. § Estimated from covariance.

roughness. For a linear fractal function, the Hausdorf–Besicovitch dimension D may vary between 1 (completely differentiable) and 2 (so rough and irregular that it effectively takes up the whole of a two-dimensional topological space). For surfaces, the corresponding range for D lies between 2 (absolutely smooth) and 3 (infinitely crumpled). Because the degree of roughness of spatial data is important when trying to make interpolations from point data such as by least-squares fitting or kriging², it is worth examining them beforehand to see if the data contain evidence of variation over different scales, and how important these scales might be. Mandelbrot's work¹ suggests that the fractal dimensions of coastlines and other linear natural phenomena are of the order of $D = 1.2$ – 1.3 , implying that long range effects dominate. I show here that published data on many environmental variables suggest that not only are they fractals, but that they may have a wide range of fractal dimensions, including values that imply that interpolation mapping may not be appropriate in certain cases.

Berry and Lewis³ have shown that the Weierstrass–Mandelbrot fractal function (WMF)

$$W(t) = \sum_{n=-\infty}^{\infty} \frac{[(1 - e^{i\gamma^n t}) e^{i\phi_n}]}{\gamma^{(2-D)n}}$$

($1 < D < 2$, $\gamma > 1$, ϕ_n = arbitrary phases)

has a power spectrum $P(\omega)$ that varies approximately as $\omega^{-(5-2D)}$, and a variance of increments $V(t) = \langle [W(t_0) - W(t_0 + t)]^2 \rangle$ that varies as t^{4-2D} at the origin. If $D > 1.5$, $V(t)$ is itself a fractal function. These results allow us to

estimate the fractional dimension D of a real series, either from the slope of the log–log plot of the power spectrum as $t \rightarrow 0$, or from

$$\frac{d \log V(t)}{d \log t} = 4 - 2D \quad (t \rightarrow 0)$$

The variance of increments (or the half thereof, the semi-variance) is much used in geostatistical studies where, computed over distances, it is referred to as the variogram². Computing the variogram is the first step in the interpolation procedure known as kriging which is used to assist estimation of mineral reserves, contouring groundwater surfaces, and so on. Thus many published data are available in this form and it is then a simple matter to calculate their dimensions D relative to the sampling interval used.

For a second-order stationary series, the variance of increments at a given lag is equal to twice the difference between the variance of the series and the covariance. Thus, D values may also be computed from covariances. In agriculture and soil science, many data have been published in the form of block variances; that is the variance within blocks of equal size plotted against block size. Because Yates⁴ showed that the variance for a block of a given size k is

$$s_k^2 = \frac{2}{k(k+1)} \sum_{h=1}^k (k-h+1) Vh$$

where s_k^2 is the block variance of block length k , and Vh the variance increment for lag h , the slope of $\log Vh$ versus $\log h$ ($h \rightarrow 0$) can also be estimated from these data.

Table 1 presents a selection of data so analysed, giving the study location, the type of environmental variable, the lag interval used for sampling and the estimated D value assuming that the real data are but a series of regularly spaced samples of a realization of the Weierstrass–Mandelbrot function over one-dimensional space or time.

The data support Mandelbrot's¹ assertion that D values of landscape and other data may range over many values. It is evident that most of the values reported here exceed 1.5, and many are greater than 1.8. Note that one of the smoothest surfaces imaginable in a landscape, a new airport runway, has a relatively high D , presumably because variations over long distance are low in amplitude. These data do not conform to a single roughness model as proposed by Sayles and Thomas,⁵ indeed, as Berry and Hannay⁶ have commented, much wider ranges of roughness or randomness are to be expected.

These results should not be accepted uncritically, however. First, it is important to realize that the Weierstrass–Mandelbrot function is just one of a class of 'model' fractals. Its peculiarity is that it has a discrete and geometric spectrum, and this might make some of its properties non-universal among other fractals with the same D (M. V. Berry, personal communication).

Second, although some environmental data do appear to display the fractal property of statistical self-similarity at all scales, there are also many that show self-similarity over a limited range of scales, or over a few widely separated scales. For example, a variable with a highly regular spatial variability has a variogram that exhibits parabolic behaviour near the origin². This is a good example where $d \log V(h)/d \log h$ becomes less steep as $h \rightarrow 0$, and it indicates that the ultra-short, short and middle range variations are trivial compared with the main variations seen at larger scales. In fact, computing D as $h \rightarrow 0$ will in this case estimate D for the ultra-short range scales of variation.

Third, the form of the variogram is often highly dependent on sampling direction and sampling interval. It is well known that when a particular sample spacing tends to match the scale of a spatial pattern, the variance of increments can fall rapidly. Accordingly, when a sampling interval matches a particular scale of a phenomenon in the landscape it perceives an apparently lower D . If this is so, it would seem more appropriate to estimate D values from the parts of the variogram showing maximum slope. Table 1 also contains these data for those phenomena showing a maximum slope at positions other than $h \rightarrow 0$.

The results presented here suggest that Mandelbrot's D can be used as a useful indicator of the complexity of autocorrelations over many scales for natural phenomena. However, although many natural phenomena do display certain degrees of statistical self-similarity over many spatial scales, there are others that seem to be structured and have their levels of variability clustered at particular scales. This behaviour does not exclude them from the fractal concept. Mandelbrot¹ considers that it is quite acceptable to have a series of zones of distinct dimensions connected by transition zones. If this is reasonable, it means that the examination of D values would be useful for trying to separate scales of variation that might be the result of particular natural processes. Moreover, identifying such scales could be of enormous practical value because one could then tailor sampling to a particular scale range of the phenomenon in question, thereby improving the efficiency of expensive field investigations and the resulting interpolations. The high level of the D values for some soil and geological data reported here would seem to question the wisdom of interpolation mapping in certain instances, however, and it would seem worthwhile to use D values as a guide to how further mapping and interpolation should proceed.

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Reactor-released radionuclides in Susquehanna River sediments

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Three Mile Island (TMI) and Peach Bottom (PB) reactors have introduced ^{137}Cs , ^{134}Cs , ^{60}Co , ^{58}Co and several other anthropogenic radionuclides into the lower Susquehanna River. Here we present the release history for these nuclides (Table 1) and radionuclide concentration data (Table 2) for sediment samples collected in the river and upper portions of the Chesapeake Bay (Fig. 1) within a few months after the 28 March 1979 loss-of-coolant-water problem at TMI. Although we found no evidence for nuclides characteristic of a ruptured fuel element, we did find nuclides characteristic of routine operations. Despite the TMI incident, more than 95% of the total ^{134}Cs input to the Susquehanna has been a result of controlled low-level releases from the PB site. ^{134}Cs activity released into the river is effectively trapped by sediments with the major zones of reactor-nuclide accumulation behind Conowingo Dam and in the upper portions of Chesapeake Bay. The reported distributions document the fate of reactor-released radionuclides and their extent of environmental contamination in the Susquehanna–Upper Chesapeake Bay System.

During the past 14 yr, five nuclear power reactors have operated for varying lengths of time at two sites on the Susquehanna River (Fig. 1). The first reactor, PB 1, was a 40-MW, gas-cooled reactor and operated from March 1966 to October 1974. Three others, PB units 2 and 3 (boiling-water reactors), and TMI unit 1 (a pressurized-water reactor), began power production in 1974 and each produces ~1,000 MW. TMI unit 2, a twin of TMI 1, was activated on 30 December 1978 but shut down on 28 March 1979 after the loss-of-coolant incident. As with the other nuclear power stations, minor amounts of ^{137}Cs , ^{134}Cs , ^{60}Co , ^{58}Co and other radionuclides are released with the coolant-water effluent. The radionuclide release history of these reactors has been compiled using Nuclear Regulatory Commission documents¹ and is summarized in Table 1. The Peach Bottom plant has contributed most of the reactor-produced radionuclides introduced into the Susquehanna River, and from 1975 to 1979, the PB reactors have released >95% of the total ^{134}Cs input to the river.

In addition to reactor releases ^{137}Cs (half life of ~30 yr) has been introduced into the Susquehanna–Chesapeake Bay system as global fallout from atmospheric nuclear weapons testing. The major influx of fallout ^{137}Cs occurred between 1962 and 1964. Reil² used the distribution of fallout ^{137}Cs in Chesapeake Bay to study the hydrography of the estuary. Although other fission products and neutron activation nuclides, such as ^{134}Cs , ^{60}Co and ^{58}Co are also produced during weapons tests, their short half lives (~2, 5 and 0.2 yr, respectively) and low yields cause the

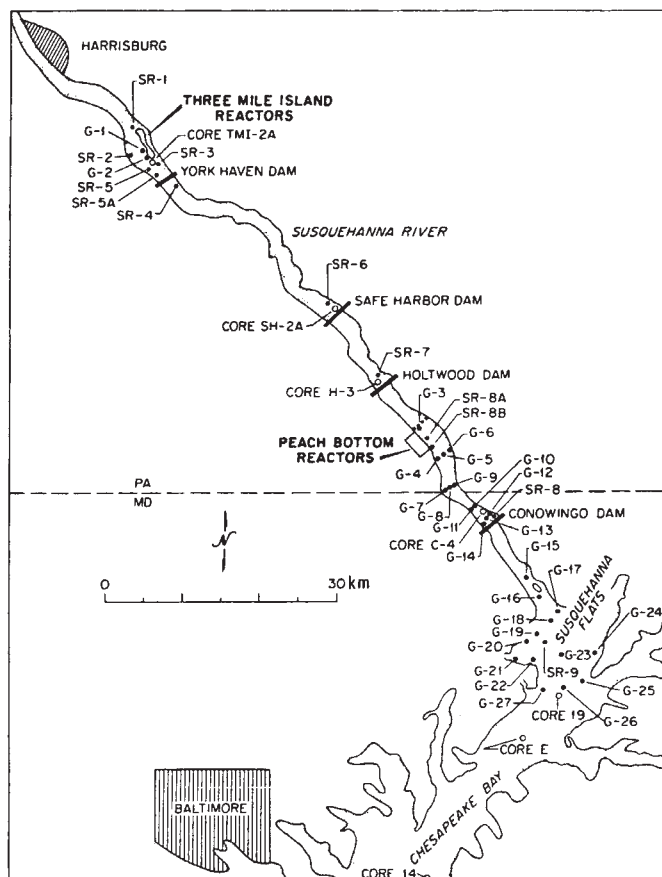


Fig. 1 Location map for sediment samples collected in the Susquehanna River and upper portions of the Chesapeake Bay. Samples labelled SR are surface sediment grabs collected in May 1979 and those labelled G are grabs collected in September 1979. ○, Core samples.

fallout contribution of these nuclides to be negligible. Consequently, observed ^{134}Cs , ^{60}Co and ^{58}Co activities in Susquehanna River sediments are a result of reactor releases only. Previous studies in the River Esk^{3,4}, Hudson Estuary⁵⁻⁷, Columbia River Estuary^{8,9}, James River Estuary¹⁰ and Chesapeake Bay¹¹⁻¹³ have shown the usefulness of these particle-associated radionuclides as tracers for sediment transport and accumulation patterns and as tools for understanding river estuarine sedimentary processes.

Radionuclide activities were measured in surface sediment samples collected from the Susquehanna River and Upper Chesapeake Bay in May and September 1979. The sediment area sampled by each grab was $\sim 15 \times 20$ cm and the sample depth ~ 10 cm. The sediments were dried, ground with a mortar and pestle, and sealed in 100-cm³ aluminium cans or 550-cm³ Marinelli beakers depending on the sample size. The activities of ^{137}Cs , ^{134}Cs , ^{60}Co , ^{58}Co and ^{40}K (a naturally occurring radionuclide) were measured using a lithium-drifted germanium detector and multichannel analyser. The activity of naturally occurring ^{40}K reflects the potassium content of the sediment (0.012% of the potassium in K-bearing minerals is ^{40}K) and correspondingly, quartz-rich sandy sediments or organic-rich peaty sediments are characterized by low ^{40}K activities.

Bomb- and reactor-produced ^{137}Cs was detected in all surface sediment samples and activities ranged from 70 to 2,500 pCi kg⁻¹ (Table 2). The highest activities of reactor-produced ^{60}Co and ^{58}Co (a few hundred pCi kg⁻¹) were detected in sediment samples near the TMI reactors. Although measurable activities (100–200 pCi kg⁻¹) of reactor produced ^{134}Cs were also observed in the sediments near the TMI reactors (Table 2), the highest ^{134}Cs activities (400–1,680 pCi kg⁻¹) were observed in the sediments near the PB reactors and just downstream behind Conowingo Dam (Fig. 2). We found no evidence in the river sediments for γ -emitting radionuclides which would

Table 1 Annual releases of radionuclides in the liquid effluent from Three Mile Island and Peach Bottom reactor sites

Year	^{134}Cs (Ci)	^{137}Cs (Ci)	^{60}Co (Ci)	^{58}Co (Ci)
Three Mile Island				
1975	<0.001	0.003	0.004	0.052
1976	0.006	0.012	0.006	0.066
1977	0.058	0.075	0.005	0.080
1978	0.145	0.019	0.016	0.481
1979	0.025	0.062	0.018	0.095
Total	0.23	0.15	0.05	0.77
Peach Bottom				
1975	<0.001	0.006	0.018	0.048
1976	0.913	0.830	0.029	0.010
1977	1.370	1.290	0.125	0.010
1978	2.860	0.810	0.154	0.027
1979	3.92	3.26	0.167	0.024
Total	9.06	6.20	0.49	0.12

Effluent release data reports from US Nuclear Regulatory Commission documents.

characterize a rupture of the fuel elements (such as ^{95}Zr – ^{95}Nb , ^{140}Ba – ^{140}La , ^{141}Ce , ^{144}Ce – ^{144}Pr , ^{103}Ru and ^{106}Ru – ^{106}Rh). Table 2 shows that the gamma activities of reactor-released radionuclides in the sediments of the Susquehanna–Chesapeake Bay System are one to two orders of magnitude less than that of naturally occurring ^{40}K (Table 2). Concentrations of naturally occurring uranium and thorium decay-series radionuclides also greatly exceed the reactor products in our samples.

Vertical distributions of radiocaesium in our sediment cores are illustrated in Fig. 3. Cores SH-2A, H-3 and C-4 were collected in May 1979 from behind the Safe Harbour, Holtwood and Conowingo Dams respectively (Fig. 1), and each was ~ 80 cm long. Box Cores 14 and 19 (each ~ 40 cm long) were collected in Upper Chesapeake Bay in August 1979. Gravity Core E was taken within the turbidity zone of Upper Chesapeake Bay (Fig. 1) in October 1976.

Although measurable activities of ^{137}Cs were observed throughout the entire lengths of cores H-3, C-4, E and 14, the activity of reactor-produced ^{134}Cs was generally confined within the top 10–15 cm (Fig. 3). The deepest appearance of ^{137}Cs in the sediment core indicates the beginning of significant fallout in

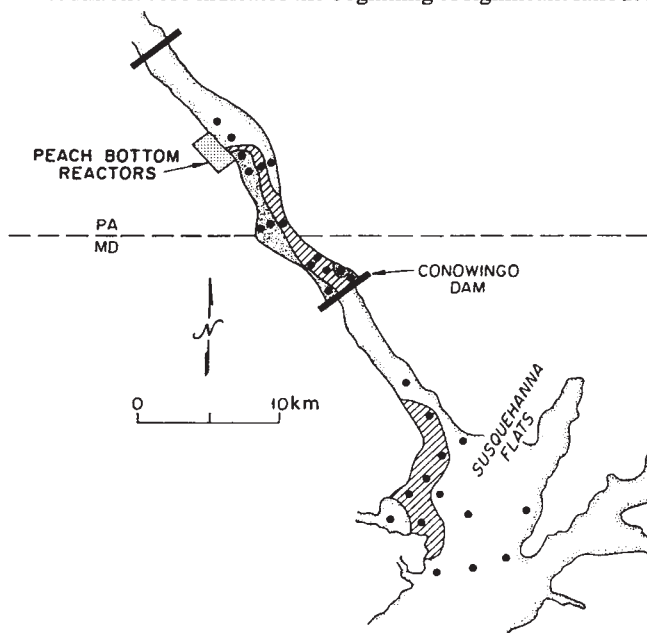


Fig. 2 Areal distribution of ^{134}Cs activity in the surface sediments downstream of the Peach Bottom reactor site. All the samples, including those taken in sandy areas of Upper Chesapeake Bay, contained detectable activities of ^{134}Cs (Table 2). The surface sediment ^{134}Cs activities are kg⁻¹: ▨, > 400 pCi kg⁻¹; ▩, 150–400 pCi kg⁻¹; □, < 150 pCi kg⁻¹.

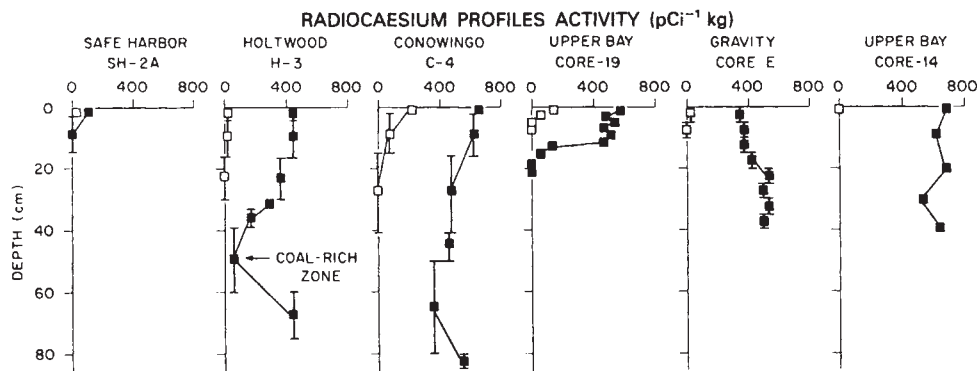


Fig. 3 Vertical distributions of radiocaesium in sediment cores indicate that the areas behind Holtwood and Conowingo Dams and areas within the turbidity zone of Upper Chesapeake Bay are sites of rapid sediment and radionuclide accumulation. Statistical counting errors are approximately the same size as the symbol ($\square$, ^{134}Cs ; $\blacksquare$, ^{137}Cs) illustrating the data point.

1954, and the deepest appearance of ^{134}Cs indicates the beginning of significant releases from the TMI and PB reactors. In addition, peaks in the radionuclide profiles may be correlated with the history of fallout or reactor releases to provide additional time-stratigraphical reference levels. Any rigorous attempt at obtaining sediment accumulation rates using these time-stratigraphical markers requires information concerning variations in sediment properties (such as grain size) and the extent of sediment mixing.

Table 2 Reactor-released radionuclides in Susquehanna River surface sediments

Location	^{137}Cs (pCi kg $^{-1}$)	$^{134}\text{Cs}^*$ (pCi kg $^{-1}$)	$^{60}\text{Co}^*$ (pCi kg $^{-1}$)	$^{58}\text{Co}^*$ (pCi kg $^{-1}$)	$^{40}\text{K}^\dagger$ (pCi kg $^{-1}$)
Three Mile Island					
SR-1	170 $\pm$ 9	11 $\pm$ 8	3 $\pm$ 7	3 $\pm$ 5	7,010 $\pm$ 240
SR-2	465 $\pm$ 16	125 $\pm$ 11	165 $\pm$ 10	485 $\pm$ 29	9,710 $\pm$ 340
SR-3	280 $\pm$ 10	2 $\pm$ 6	10 $\pm$ 10	1 $\pm$ 7	8,370 $\pm$ 300
SR-4	175 $\pm$ 8	16 $\pm$ 4	4 $\pm$ 7	23 $\pm$ 6	8,480 $\pm$ 260
SR-5	635 $\pm$ 22	105 $\pm$ 12	255 $\pm$ 13	365 $\pm$ 25	15,590 $\pm$ 520
SR-5A	620 $\pm$ 23	185 $\pm$ 16	170 $\pm$ 14	310 $\pm$ 24	10,840 $\pm$ 420
G-1	470 $\pm$ 25	17 $\pm$ 5	2 $\pm$ 5	—	14,610 $\pm$ 760
G-2	620 $\pm$ 35	83 $\pm$ 13	51 $\pm$ 6	—	12,090 $\pm$ 660
Safe Harbor Dam					
SR-6	765 $\pm$ 26	20 $\pm$ 9	9 $\pm$ 16	—	18,720 $\pm$ 620
Holtwood Dam					
SR-7	395 $\pm$ 15	67 $\pm$ 10	8 $\pm$ 12	—	15,440 $\pm$ 490
Peach Bottom					
SR-8A	795 $\pm$ 17	15 $\pm$ 5	1 $\pm$ 8	—	18,260 $\pm$ 510
SR-8B	2,460 $\pm$ 48	1,680 $\pm$ 86	180 $\pm$ 10	28 $\pm$ 9	11,820 $\pm$ 390
G-3	110 $\pm$ 9	13 $\pm$ 5	1 $\pm$ 4	—	8,230 $\pm$ 450
G-4	1,280 $\pm$ 67	870 $\pm$ 94	160 $\pm$ 10	—	9,880 $\pm$ 530
G-5	210 $\pm$ 14	115 $\pm$ 14	2 $\pm$ 5	—	6,260 $\pm$ 360
G-6	1,890 $\pm$ 100	79 $\pm$ 14	5 $\pm$ 9	—	15,930 $\pm$ 870
G-7	535 $\pm$ 31	415 $\pm$ 46	10 $\pm$ 5	—	7,790 $\pm$ 440
G-8	850 $\pm$ 47	580 $\pm$ 64	40 $\pm$ 8	—	9,780 $\pm$ 550
G-9	315 $\pm$ 17	180 $\pm$ 20	9 $\pm$ 4	—	5,840 $\pm$ 300
Conowingo Dam					
SR-8	635 $\pm$ 22	130 $\pm$ 13	12 $\pm$ 15	ND	19,530 $\pm$ 630
G-10	535 $\pm$ 32	220 $\pm$ 26	8 $\pm$ 7	—	16,230 $\pm$ 880
G-11	670 $\pm$ 36	210 $\pm$ 24	6 $\pm$ 6	—	14,920 $\pm$ 790
G-12	1,070 $\pm$ 58	490 $\pm$ 55	29 $\pm$ 9	—	18,480 $\pm$ 990
G-13	710 $\pm$ 44	160 $\pm$ 24	8 $\pm$ 14	—	16,010 $\pm$ 920
G-14	960 $\pm$ 51	450 $\pm$ 48	22 $\pm$ 5	—	16,420 $\pm$ 860
Susquehanna Flats					
SR-9	195 $\pm$ 11	110 $\pm$ 10	3 $\pm$ 11	—	11,600 $\pm$ 370
G-15	190 $\pm$ 10	145 $\pm$ 15	2 $\pm$ 2	—	4,610 $\pm$ 240
G-16	390 $\pm$ 22	280 $\pm$ 32	4 $\pm$ 4	—	5,080 $\pm$ 290
G-17	135 $\pm$ 10	105 $\pm$ 13	2 $\pm$ 7	—	13,520 $\pm$ 710
G-18	225 $\pm$ 12	160 $\pm$ 18	2 $\pm$ 3	—	7,950 $\pm$ 410
G-19	330 $\pm$ 17	265 $\pm$ 21	2 $\pm$ 2	—	3,940 $\pm$ 210
G-20	445 $\pm$ 23	275 $\pm$ 30	5 $\pm$ 2	—	5,490 $\pm$ 280
G-21	79 $\pm$ 8	29 $\pm$ 7	8 $\pm$ 7	—	11,770 $\pm$ 630
G-22	790 $\pm$ 43	330 $\pm$ 37	8 $\pm$ 8	—	12,240 $\pm$ 660
G-23	440 $\pm$ 26	140 $\pm$ 17	6 $\pm$ 7	—	9,290 $\pm$ 510
G-24	76 $\pm$ 5	27 $\pm$ 3	3 $\pm$ 2	—	2,350 $\pm$ 130
G-25	480 $\pm$ 26	43 $\pm$ 8	7 $\pm$ 7	—	14,350 $\pm$ 750
G-26	480 $\pm$ 27	100 $\pm$ 13	7 $\pm$ 7	—	11,940 $\pm$ 640
G-27	175 $\pm$ 13	58 $\pm$ 10	4 $\pm$ 8	—	14,260 $\pm$ 760

ND, not determined.

* Activities decay corrected to date of sample collection, 22 May 1979 for SR samples and 26 September 1979 for G samples.

† Natural ^{40}K activities $> 10,000$ pCi kg $^{-1}$ indicate muddy samples.

‡ Dashes indicate no ^{58}Co data, because the date of analysis was more than three half lives (273 days) after the date of collection.

Our radiocaesium profiles (Fig. 3) indicate that the areas behind Holtwood and Conowingo Dams are presently zones of rapid sediment and radionuclide accumulation. Gross *et al.*¹⁴ concludes that one-half to two-thirds of the sediment load of the lower Susquehanna is trapped behind the three hydroelectric dams and that $\sim 1 \times 10^6$ Mg of suspended sediment are annually discharged past Conowingo Dam during years without major floods. The low activities for reactor radionuclides and the lack of radiocaesium in the core collected behind Safe Harbour Dam indicate that either this reservoir has not been a major trap for fine particles and radionuclides during the past two decades or our samples are not representative of the area as a whole. A detailed budget of sediment accumulation behind the dams based on radionuclide data from these and several more recent cores, is in preparation (J.F.D and O.P.B.).

The sediment and radionuclides which escape from behind Conowingo Dam seem to be efficiently trapped along the western margin of the Susquehanna Flats (Fig. 2) or in the turbidity maximum area (Core E) of Upper Chesapeake Bay. Goldberg *et al.*¹³ have observed sediment accumulation rates as great as 8 cm yr $^{-1}$ (and $^{239,240}\text{Pu}$ activity distributions to sediment depths as great as 70 cm) in the turbidity zone area near the location of Gravity Core E. The elevated PCB concentrations (0.1 p.p.m.) at 35–40 cm (ref. 15) in Core E and our ^{137}Cs profile support the conclusions of Goldberg *et al.*¹³ that during the past two decades this area has been a zone of rapid sediment and contaminant accumulation.

The depth of ^{137}Cs penetration in Core 14, collected near Baltimore (Fig. 1), indicates that this may also be an area of recent particle accumulation. The lack of ^{134}Cs activity in the surface sediments of this core indicates that Susquehanna-derived particles at this site are markedly diluted by sediments from other sources.

As most of the ^{134}Cs activity was only recently released from the PB reactor site, and because much of this ^{134}Cs activity seems to be confined to near the sediment surface (Fig. 3), we can estimate the total ^{134}Cs burden in the sediments by integrating the ^{134}Cs activities in our grab samples and using these values as representative of different areas in the river and upper bay. A detailed budget of ^{134}Cs accumulation, based on more samples than those presented here, is in preparation but preliminary estimates indicate that almost all the ^{134}Cs released into the Susquehanna River has been trapped within the sediments upstream of Baltimore¹⁶.

Our distribution data indicate that: (1) reactor-produced radiocobalt and radiocaesium are sequestered by particles and deposited in low-energy environments; (2) levels of contamination, though measurable, are a small fraction of the total radioactivity resulting from naturally occurring radionuclides; and (3) these nuclides can provide valuable insights into the transport and accumulation patterns of particles and particle-associated pollutants introduced into the Susquehanna–Upper Chesapeake Bay system.

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Field-capacity water extracts from serpentine soils

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Soils derived from serpentinites and other ultramafic rocks have long been called 'serpentine soils' by biologists¹. They often bear a distinctive vegetation with poor cover or rare species or both and have attracted botanical interest for centuries^{2,3}. The chemical causes of the unusual vegetation have often been investigated using soil analyses of exchangeable and total quantities of ions or elements. These have shown that serpentine soils are rich in magnesium and have relatively high concentrations of nickel but low ones of calcium and other nutrients. Although these factors have been judged to be important in many serpentine soils, there have been few analyses of their soil solutions^{4–6}, which would give a better assessment of likely soil toxicities and deficiencies. Moreover the soil solution can be effectively simulated in water-culture experiments to investigate directly the effect of each possible factor on plant growth. We report here (Table 1) the results of soil-solution analyses for a range of Scottish and Zimbabwean serpentine soils which bear unusual vegetation. These analyses have substantially altered previous conclusions concerning the chemical causes of serpentine vegetation⁷.

The soil solutions were extracted by centrifuging at 12,000g soil samples which had been maintained for up to 3 days at field capacity (moisture content after 24 h drainage). The solutions

were analysed by standard atomic absorption techniques⁸ for metals, the phenol disulphonic method⁹ (with modification in high chloride samples) for nitrate, the molybdenum blue method for phosphorus⁹ and by a silver titration method for chloride⁹. pH was measured using a glass electrode and bench pH meter.

The results are best discussed in the context of the three main chemical causes of plant growth on serpentines: high Mg/Ca quotient, high nickel and low nutrients.

The Meikle Kilrannoch soil is apparently uniquely toxic among British serpentines (J.P., unpublished results, and ref. 10) and there is evidence^{5,10–12} to indicate that high magnesium is the prime cause of this toxicity. Magnesium toxicity depends on the concentration of calcium and, from soil exchangeable-cation analyses it seemed that very low calcium exacerbates magnesium toxicity at Meikle Kilrannoch¹¹. The soil-solution analyses (Table 1) offer a different explanation. The distinctive feature of the Meikle Kilrannoch soil solution is its high concentration of ions, particularly magnesium among the cations. Calcium is actually at higher concentrations than in other Scottish serpentines (in Table 1) but proportionally more calcium is required to ameliorate magnesium toxicity at higher solution concentrations of this element^{5,12,13}.

The soil-solution analyses have also been useful in judging the possibility that calcium deficiency may be an adverse factor in some serpentines. Several earlier workers had stressed the importance of calcium deficiency^{14,15} but the demonstration of the very small absolute requirement for calcium by plants¹⁶ had suggested that calcium deficiency was unlikely to occur in serpentine soils. The lower soil-solution calcium concentrations at Coyles of Muick, Hill of Towanreef and particularly Green Hill (sites where soil exchangeable calcium is not very low^{17,18}) still exceed the minimum calcium required by *Zea mays*¹⁶. The possibility of calcium deficiency as a plant-determining factor in some serpentine soils should be reconsidered. Very low soil-solution calcium at Green Hill has been observed⁶ and three species of grass tolerant to this soil have root-surface

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Table 1 The means ($\pm 95\%$ confidence limits) of ions (mg l^{-1}), pH and Mg/Ca quotients in soil solutions in skeletal soils from serpentine sites in Scotland and Zimbabwe

Sites	No. of samples	Ni ²⁺	Ca ²⁺	Mg ²⁺	K ⁺	Na ⁺	Cl ⁻	NO ₃ ⁻	PO ₄ ³⁻ #	pH	Mg/Ca
Scotland											
Coyles of Muick**	10	<0.1	0.80 $\pm$ 0.21	9.1 $\pm$ 1.2	0.30 $\pm$ 0.07	4.5 $\pm$ 0.74	3.1 $\pm$ 0.59	52 $\pm$ 11	1.3 $\pm$ 1.0	6.8 $\pm$ 0.15	13 $\pm$ 3.7
Green Hill**	8	<0.1	0.45 $\pm$ 0.12	9.4 $\pm$ 1.8	0.36 $\pm$ 0.13	5.1 $\pm$ 0.99	4.2 $\pm$ 0.66	50 $\pm$ 12	0.95 $\pm$ 0.92	6.5 $\pm$ 0.21	23 $\pm$ 8.1
Hill of Towanreef**	10	<0.1	0.80 $\pm$ 0.31	12 $\pm$ 3.9	0.58 $\pm$ 0.29	9.6 $\pm$ 6.6	8.3 $\pm$ 9.5	57 $\pm$ 7.6	3.2 $\pm$ 1.7	6.6 $\pm$ 0.18	18 $\pm$ 7.1
Keen of Hamar (Unst)*†‡	11	0.13 $\pm$ 0.04	5.4 $\pm$ 2.3	28 $\pm$ 15	5.0 $\pm$ 1.6	110 $\pm$ 3.2	200 $\pm$ 72	29 $\pm$ 14	0.97 $\pm$ 0.10	5.8 $\pm$ 0.26	5.0 $\pm$ 0.66
Meikle Kilrannoch†§	11	0.67 $\pm$ 0.069	11 $\pm$ 1.2	180 $\pm$ 33	5.4 $\pm$ 1.2	15 $\pm$ 1.4	ND	830 $\pm$ 69	13 $\pm$ 5.0	ND	ND
Zimbabwe											
Kingston Hill ¶	4	0.58 $\pm$ 0.48	19 $\pm$ 9.8	28 $\pm$ 21	20 $\pm$ 10	ND	ND	ND	ND	ND	1.5 $\pm$ 0.36
Tipperary Claims ¶	3	0.67 $\pm$ 0.29	23 $\pm$ 36	38 $\pm$ 56	19 $\pm$ 32	ND	ND	ND	ND	ND	1.8 $\pm$ 1.6

* Samples collected moist in August 1980, stones > 1 cm diameter removed, wetted to field capacity a few days after collection and centrifuged after 1–3 days.

† Chromium and cobalt analyses carried out on these samples: none exceeded the detection limit of 0.1 mg l^{-1} .

‡ One sample was missing from the nitrate analyses. Three samples with nickel below the detection-limit (0.1 mg l^{-1}) were not included in the calculation of mean and confidence limits for this element.

§ Samples collected moist in September 1976, sieved through a 2 mm mesh, air-dried, moistened to field capacity in laboratory in May 1977 and centrifuged after 3 days.

|| ND, not determined.

¶ Samples collected from relatively dry soils in January 1977, sieved through a 2 mm mesh, air-dried, subsequent treatment as for Meikle Kilrannoch.

Phosphate species expressed as PO₄³⁻.

** Nickel values were all below the detection limit (0.1 mg l^{-1}) except for one sample from Green Hill with 0.18 mg l^{-1} nickel.

phosphatases with activities unchanged in a range of calcium concentrations. Two grass species which grew poorly in Green Hill soil had calcium-sensitive root-surface acid phosphatases and it was inferred that the tolerant species were adapted to low soil calcium. In contrast, native *Festuca rubra* from Meikle Kilrannoch¹⁹ has root-surface phosphatases with higher activities with increasing calcium, magnesium and nickel.

The use of water-culture experiments with *Festuca rubra* in media simulating soil solutions has shown that nickel is also likely to be toxic (at $0.7 \text{ mg l}^{-1} \text{ Ni}^{2+}$), although less so than magnesium, at Meikle Kilrannoch¹³. Similar experiments with *Agrostis stolonifera* in simulated Keen of Hamar soil solutions have provided evidence of slight nickel toxicity (at $0.1\text{--}0.2 \text{ mg l}^{-1} \text{ Ni}^{2+}$)²⁰. These experiments have allowed nickel toxicity in British serpentines to be assessed and helped to resolve conflicting evidence from soil and plant analyses and bioassays (J.P. unpublished data, and refs 10, 11, 18).

Earlier work in Zimbabwean serpentine soils had indicated the importance of nickel toxicity for plant growth^{21–23}. Our soil-solution analyses support this conclusion, and show a low Mg/Ca quotient in the Zimbabwean samples, confirming that this factor is less important than a high nickel concentration.

The present results show that nutrient concentrations are not always low in serpentine soils. The nitrate concentrations at Meikle Kilrannoch are high and nitrate is the main balancing anion for magnesium at Coyles of Muick, Green Hill and Hill of Towanreef. Nitrate is lower at the Keen of Hamar. Here the site is close (all samples within 0.5 km) to the sea and the soil solutions resemble (with some enrichment of magnesium and nickel) ~1% seawater with Na^+ and Cl^- as preponderant ions. Soil-solution phosphate concentrations in Table 1 are high but for this element the labile-pool size, for which we have no information, may be a more critical determinant of plant growth. Potassium concentrations range from low in the Scottish soils to moderately high in those from Zimbabwe.

Hydrogen and hydroxyl ions must contribute little to the total ion balance in these soil solutions as they are neutral to mildly acid. Table 1 shows direct measurements for four sites: measurements of soil pH (in a mix of equal proportions of soil and water) gave mean values of 6.7 for Meikle Kilrannoch, 6.1 for Kingston Hill and 6.2 for Tipperary Claims for samples collected close to those used for the soil-solution analyses.

The relevance of soil-solution extraction at field capacity can be questioned as soils are in this state for an unknown part of the growing season. Solution concentration can vary with the time of the year and with the method of extraction^{24,25}. But cool moist conditions prevail at the Scottish sites and here the field-capacity extraction seems appropriate. Moreover, as the concentration of the soil solution increases on drying²⁴ the potentially toxic nickel in the field-capacity water extracts from the Zimbabwean samples represents a minimum level of nickel toxicity which must become much more acute. It is encouraging that *F. rubra* plants grown in water-culture media based on the field-capacity extracts from Meikle Kilrannoch soils have shown a broadly similar appearance, size and tissue element composition to the same species grown in the actual soils¹³.

Similar soil-solution analyses have been used successfully in recent studies of the vegetation of skeletal soils rich in copper, lead and zinc^{26,27}. However, the interpretation of soil-solution analyses is less straightforward for soils with much organic matter where heavy metals are likely to be in complexes of unknown chemistry and toxicity¹⁹.

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Upper Cretaceous to Eocene pelagic limestones of the Scaglia Rossa are not Miocene turbidites

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The Scaglia Rossa is a pink limestone composed of 1–20% forams and ~5% clay in a coccolith matrix that outcrops extensively in the Umbria–Marches Apennines of northern peninsular Italy. It has universally been considered to be a pelagic sedimentary rock deposited during the late Cretaceous to middle Eocene. The Scaglia Rossa has been studied intensively as a record of micropalaeontological^{1–7}, palaeomagnetic^{8–30}, sedimentological^{31–34}, and geochemical^{33,35–39} events. Recently, Wezel⁴⁰ reported that the limestones are not of pelagic origin but are entirely turbiditic, and were deposited in the Miocene. He also criticized magnetic polarity stratigraphy investigations done on the Scaglia Rossa, concluding that the polarity is a function of sedimentation rate, not of geomagnetic field polarity. His evidence has cast doubt on previous stratigraphic information from the Scaglia Rossa⁴¹ and compels us to respond. Although the observation by Coccioni⁴² and Wezel⁴⁰ of the presence of fossils of anomalously young ages in clay seams between the limestone beds is of interest, we show here that the proposed redating of the Scaglia is wrong for several palaeontological, sedimentological and palaeomagnetic reasons.

Wezel stressed the importance of Coccioni's⁴² first report of Miocene planktonic forams in clay partings between limestone beds in the Scaglia⁴⁰ but did not comment on Premoli Silva and Luterbacher's⁴³ conclusion that these younger forams were contaminants.

On checking these observations we have found that Miocene forams are indeed present in clay interbeds in various places. However, detailed examination of ~200 thin sections of Scaglia limestone beds from six localities, including 57 thin sections from the Bottaccione Gorge at Gubbio, spanning the same stratigraphic interval as Coccioni's⁴² clay samples, did not reveal a single Miocene foram, although the spherical forms *Orbulina* and *Praeorbulina*, the most common types identified by Coccioni, yield distinctive circular sections. Wezel⁴⁰ found no detrital grains accompanying the younger forams, and because these forams are the same age as the turbiditic Marnoso-arenacea Formation, which is rich in quartz and mica grains, he concluded that the Miocene forams could not be contaminants derived from the Marnoso-arenacea. However, in a test of this conclusion carried out at the University of California, A. Montanari (personal communication, 1981) washed clay from the Cretaceous–Tertiary boundary in the Bottaccione section at Gubbio and found rare quartz grains and particles of cemented

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quartz sandstone and siltstone, some of the latter actually containing Miocene planktonic forams cemented into the siltstone. He also found these materials by washing soil collected on the Scaglia Rossa outcrop face. These observations are striking proof that the Miocene forams are contaminants derived from the Marnoso-arenacea.

Wezel⁴⁰ illustrates one thin section from a hard limestone bed of the basal Palaeocene *Globigerina eugubina* zone showing a foram which he first identifies as the Upper Palaeocene to Middle Eocene planktonic form "*Globorotalia* ex gr. *G. aragonensis* - *G. formosa*" and later as "*Globorotalia* cf. *aragonensis*"; "ex gr." (out of group of) and "cf." (compare) indicate that the fossil could not be precisely identified. However, according to Premoli Silva (personal communication, 1980), the wall structure of this foram indicates that it is a benthic, not a planktonic type; it probably belongs to the genus *Conorotalites*.

The Scaglia Variegata, Scaglia Cinerea and Bisciaro units, which overlie the Scaglia Rossa, have been found to contain an orderly sequence of Upper Eocene-Lower Miocene forams and coccoliths⁴⁴⁻⁴⁷. In Wezel's interpretation all previous work on these units must be wrong.

Wezel⁴⁰ considers that the Scaglia Rossa is a Cretaceous-Palaeocene sediment redeposited by turbidity currents during the Miocene. He therefore stresses the occurrence of turbiditic beds in some sections of the Scaglia Rossa. These beds, showing grading, sole marks and Bouma sequences, and described as 'detrital beds', 'calcarenes', or 'turbidites'^{3,48-52}, are usually conspicuously white in a formation that otherwise is dominantly pink. Wezel⁴⁰ goes beyond other workers in considering other parts of the Scaglia Rossa to be of turbiditic origin. Describing the Bottaccione section at Gubbio, which all others consider to be entirely pelagic, Wezel⁴⁰ says: "Except for some lamination, calcareous beds do not seem to exhibit obvious turbidite structures. Bedding-parallel stylolites and recrystallization obliterated the possible structures." Nevertheless, he infers a "turbidite distal setting for the calcilutites of Bottaccione". He suggests that a 350-m sequence of foram-coccolith limestones, showing a normal 50 Myr sequence of foraminiferal biozones^{4,7}, is composed entirely of turbidites, when there has been no report of any turbiditic feature. A more probable conclusion is that the Scaglia Rossa is a pelagic sediment in which there are local intercalations of calcareous turbidites.

All palaeontologists who have studied the Scaglia have recognized an orderly sequence of Upper Cretaceous to Middle Eocene foraminiferal zones⁵⁻⁷. Even Wezel⁴⁰ admits that the Scaglia shows a normal "thin section biostratigraphy". If the Scaglia is Miocene, the orderly Cretaceous to Eocene foraminiferal zonation must be explained. Wezel⁴⁰ explains the gross normal 'thin section biostratigraphy' with the intervention of an intermediate area of sedimentary accumulation, located between the source area and the final basin of Scaglia Rossa deposition. This means that after deposition of a normal stratigraphic sequence of Cretaceous-Eocene pelagic carbonate ooze, successive layers were stripped off and redeposited by turbidity currents in an intermediate basin. This would invert the order of biozones. Subsequently the process was repeated, so that in the final deposit—the present-day Scaglia—the original order of biostratigraphic zones was restored (Wezel, personal communication, 1979).

Although Wezel⁴⁰ states that this is not an extravagant hypothesis, mobilization of turbidity currents simply does not occur by the stripping off of broad but very thin sheets; it is a process which inevitably mixes material from a considerable stratigraphic thickness of the source body. Yet Wezel requires that this happen twice with no increase in the disorder of the sedimentary sequence.

Five sections of Scaglia Rossa, including the Bottaccione section, have been found to contain an abnormal abundance of iridium precisely at the Cretaceous-Tertiary boundary^{35,36,39}. Similar iridium anomalies occur at the Cretaceous-Tertiary boundary in several other locations. (Denmark, at the type

Danian locality^{35,36,39,53,64}, southern Spain⁵⁴, northern Spain⁵⁵, New Zealand^{39,55}, and the north-central Pacific⁶⁴). As iridium is greatly depleted in the Earth's crust relative to its cosmic abundance, the anomaly has been taken to indicate an influx of extraterrestrial material at the time of terminal Cretaceous extinctions^{35,36,39}. If the Scaglia Rossa is really Miocene, it must be coincidence that an iridium anomaly occurs just where the reworked Cretaceous and Palaeocene forams suggest that we are seeing the Cretaceous-Tertiary boundary.

Wezel dismisses the concordant palaeomagnetic results of four independent groups who, since 1973, have measured several thousand samples, and agree that the Scaglia Rossa is an excellent recorder of the magnetic field⁸⁻³⁰. Umbrian palaeomagnetic directions from Jurassic to Palaeocene define a segment of an apparent polar wander path which, after correction for a tectonic rotation reflecting the overall geodynamic history of the region, is consistent with the reconstructed African polar wander path for that time interval²⁸.

The Scaglia Rossa has yielded a magnetic polarity record that exactly matches the sea-floor spreading magnetic anomalies in the parts of the ocean known from deep-sea drilling to be of late Cretaceous and early Tertiary age^{16,23}. Subsequently we have found in the Umbrian sequence an essentially perfect match from anomaly M-4 (Barremian) to anomaly 6C (basal Miocene), an interval of about 100 Myr (refs 29, 30, 46, 47, 63, 65). Furthermore ~80% of this reversal sequence has been checked by measuring more than one section. The same magnetic reversal sequence is found in the Southern Alps, exactly correlated with the biostratigraphic sequence from studies in the Hauterivian through the Maastrichtian³⁰. Magnetic stratigraphic studies on DSDP Legs 73 and 74 have further confirmed parts of the reversal sequence^{56,57}. The accumulated magnetostratigraphic results provide overwhelming evidence that the Scaglia Rossa is not Miocene.

Wezel's explanation of this polarity stratigraphy is that normal directions occur in thin beds whereas reversed directions occur in thick beds. His Fig. 4 shows columns giving magnetic properties and bedding thickness at Gubbio and he states that they correlate, but we fail to detect any correlation in this figure. The intensity of magnetization of a sediment depends on the concentration of magnetic minerals, which might be related to bedding thickness. However, the direction of remanence is acquired by post-depositional alignment of magnetic grains in water-logged interstices below the sediment-water interface and is independent of the magnetic mineral concentration^{10,58-61}. Therefore, even if a correlation between remanent intensity and bedding thickness were to be demonstrated, this would be unrelated to the polarity sequence.

If the Scaglia Rossa is Miocene, and if the Scaglia Bianca is correctly dated as Cenomanian (F. C. Wezel, personal communication, 1979), there must be a hiatus of ~80 Myr between the two formations. Wezel indicates that the hiatus is hard to find because the Scaglia Bianca-Scaglia Rossa sequence is paraconformable. We note that the pattern of magnetic declinations and inclinations shows no break in the vicinity of the Bianca-Rossa contact^{24,26}, which would be extremely improbable if there were a hiatus of 80 Myr hidden in the sequence. Moreover, 100 m of normally polarized limestone lie above the Scaglia Bianca-Scaglia Rossa contact^{21,23,24,26,29}. This corresponds to the later part of the Cretaceous magnetic quiet zone. There is no equivalent to this long normal zone in the Miocene reversal history⁶².

If the Scaglia Rossa is Miocene, the perfect match between the Umbrian magnetic stratigraphy and the sea-floor anomalies for almost 100 Myr must be another coincidence unless we consider that all of the sea floor currently considered to be of late Cretaceous to Oligocene age is actually of Miocene age. Many DSDP Initial Reports document that this is not the case.

It has been suggested that the Miocene forams between limestone beds at Gubbio might indicate that portions of the Scaglia Rossa are huge osteoliths. The palaeomagnetic stratigraphy shows that this is not the case. Declinations change

slowly and smoothly; they do not show the sudden breaks that should occur at the limits of olistostrome blocks, at least some of which would have rotated during emplacement. Rotations of this kind have been found in several synsedimentary slide masses 5–15 m thick in the turbidite-bearing Furlo section⁶³; they are not present at Gubbio, or in any of the entirely pelagic sections which have been studied.

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A reply by F. C. Wezel

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I thank Alvarez and Lowrie for their comments on my paper on the Scaglia Rossa¹. However, I found their comments scientifically unsatisfactory for at least two reasons. First, although my work was published two years ago they present no new observed facts. Rather they seem to have adopted a model to which all data must be fitted. Second, although I raised the problem of Miocene foraminifera in the Scaglia Rossa turbidites, I did not, in fact, conclude that they were Miocene in age.

Further, Alvarez and Lowrie seem to have misinterpreted the spirit of my paper. In dispelling the alleged cliché of the Scaglia Rossa as "a conformable, complete and pelagic sequence of oceanic type"^{2,3}, I stressed the rudimentary state of our factual geological knowledge of the formation and the urgent need for

more research "before continuing to pursue other striking speculations remote from facts"¹. It is regrettable that Alvarez and Lowrie have ignored some basic points of my paper contradicting the inferred pelagic origin of the Scaglia Rossa limestones. For example, (1) The palaeogeographic reconstruction indicating a series of narrow and current-influenced troughs in a probable taphrogenic regime, marked by a combination of subsidence and distension. (2) The facies and palaeocurrent analysis showing the distal basinal environment of the Bottaccione section. (3) The basin-wide regional megarhythmicity of the vertical sequences, expressing temporal changes in sedimentation rate as a consequence of the structural activity of the passive margin tectonics.

The micropalaeontological data of Coccioni (personal communication) clearly show the presence of Miocene foraminifera in clay interbeds from various sections of the Scaglia Rossa. This work was carried out in our laboratory by painstakingly washing 400 kg of sample; 238 Miocene foraminiferal tests were recovered. Alvarez and Lowrie's statement that "not a single Miocene foram was found . . . in detailed examination of about 200 thin sections of Scaglia limestone beds from six localities . . .", is not at all surprising. It rather pinpoints the methodological inadequacy of thin section studies. The presence of Miocene microfossils represents undoubtedly a real problem which has not yet been solved. With our current knowledge, I am now inclined to believe that the Scaglia Rossa is probably Cretaceous–Eocene in age, but complete disregard of the observed younger foraminifera is not an adequate solution to the problem.

Alvarez and Lowrie did not take into account a vast fund of geological information. They state once more the presence of an anomalously high iridium concentration at the Cretaceous–Tertiary boundary in the Bottaccione and other four sections of Scaglia Rossa. They stated that "No iridium anomaly is yet known at any other level in any pelagic sediment". Possibly one could believe in such "iridium anomaly" after many analyses have been undertaken in other beds of the sedimentary column (see ref. 4). Unfortunately, however, the concerted effort of the catastrophists^{5–7} focused almost exclusively on the 'magic' thin Cretaceous–Tertiary boundary clay. In such conditions, without a thorough and detailed examination of numerous other clay interbeds and consideration of the minero-petrographic and geochemical contexts, it would be wrong to interpret a unique event as a catastrophe.

Some very significant recent results have now demonstrated other iridium anomalies at different levels. For example, a series of analyses carried out on the 1-m thick cherty black shale ('Bonarelli level', considered Turonian in age), located ~240 m under the Cretaceous–Tertiary boundary, has unequivocally shown an anomalously high iridium concentration, about twice as great as the K/T clay⁸. Recent geochemical and petrographic investigations⁹ suggest that this iridium anomaly could be imputed to volcanic activity and not to a second even more drastic extraterrestrial catastrophe.

Finally, I should like to re-emphasize the crude relationship I observed between magnetic properties and variations in lithology. Palaeomagnetic data from the thick coarser-grained turbidites may be controlled by depositional currents, whereas those from the finer-grained beds may more accurately record the Earth's magnetic field.

Therefore the conclusions by Alvarez and Lowrie do not seem to be supported by adequate facts and thus the 65-Myr catastrophic event may be considered an imaginative but unproven hypothesis. I therefore urge, once again, a critical assessment of the different aspects of the Scaglia Rossa geology.

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Population trends among Jamaican reef corals

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Disturbance has been cited as a potentially important agent in structuring ecological communities by modifying the effects of competition¹⁻⁵. Catastrophic disturbance has also been proposed as a factor promoting the coexistence of competing species in highly diverse tropical ecosystems such as rain forests and coral reefs^{3,6-11}. Here we describe patterns of recruitment and mortality among reef corals over 4 yr at several depths on the reefs of Discovery Bay, Jamaica, which were struck by Hurricane Allen on 6 August 1980¹². Photographic quadrats monitored since 1976 on a shallow water reef showed a negative correlation between coral abundance and mortality which was not offset by compensatory patterns of recruitment. This slow trend in the disproportionate reduction of rarer, competitively inferior species was reversed by Hurricane Allen, with storm-induced mortality being greatest in the most abundant species. On deeper reef stations, undisturbed by the storm, slower rates of colony loss were compensated for by commensurate rates of colony recruitment. Thus, patterns of differential mortality and recruitment contribute to the maintenance of high species diversity in this tropical marine ecosystem.

Photographic stations were established on Monitor Reef, on the West Fore Reef of Discovery Bay, a well studied area¹³ of classic Caribbean reef morphology^{14,15}, in September 1976, and were rephotographed biennially until 1980 (Fig. 1). Photographs were analysed for per cent cover, species number and relative abundance within the plots, recording the fates of 1,718 individual coral colonies in 24 species with respect to recruitment, whole and partial mortality, and growth.

Reef-building corals are photosynthetic and compete to secure space for the interception of both light and plankton¹⁶⁻¹⁹. Competitive outcomes between neighbouring individuals are determined by differential growth rates, overtopping morphologies and abilities in extra-coelenteric digestion^{2,7,20-22}. Before Hurricane Allen, mortality among shallow water corals from photostations II (3.8 m depth) and IV (6.7 m depth) was inversely density dependent, with species of lesser abundance suffering proportionally greater percentages of loss due to partial as well as complete mortality of individual colonies (Fig. 2). Recruitment rates were low for all species in shallow water stations; recruitment rates to photostations II, IV and XII of newly arriving colonies which achieved a size of $\geq 1 \text{ cm}^2$ ranged from 0.0 to $1.81 \text{ m}^{-2} \text{ yr}^{-1}$, with an average rate for all 24 species in the 4-yr survey before Hurricane Allen of $0.19 \pm 0.39 \text{ m}^{-2} \text{ yr}^{-1}$. In addition, the disproportionate rate of loss among the rarer species was not offset by compensatory recruitment patterns. These data suggest that in the absence of a major perturbation, there is a tendency for shallow-water communities to become monopolized by the branching *Acropora* species, causing a reduction of diversity with time.

Hurricane Allen was the second strongest recorded in the New World this century^{12,23}. It was designated as a 5 on the International Hurricane Scale (there is no 6), with central pressures of 899 mbar and maximum wind speeds of 285 km h^{-1} (ref. 24). The north coast of Jamaica, normally sheltered from severe storms²⁵, received storm waves ranging from 6 m height over the phototranssect area to 12 m height on less sheltered

reefs on the eastern side of the bay. (The last hurricane with similar track to Allen was in 1917²⁶. However, disturbance sufficient to set back *Acropora* dominance may occur in severe winter storms at greater frequency.) Damage to corals varied with their depth of attachment, exposure to impinging waves, skeletal tensile strength and morphology²³. Analysis of the storm effects in the phototranssect areas reveals that in water less than 10 m deep, mechanical disturbance from fracture, scour and toppling reduced living coral cover from 51–54% to 10–12%. Mortality was greatest among the branching, friable and loosely attached *Acropora* species²⁷⁻²⁹ which numerically and areally dominated the pre-hurricane reef. During the first 4 yr of the survey, we documented an increase in dominance by these species, a trend which was reversed during Hurricane Allen (Fig. 2). This reversal in mortality patterns does not result from

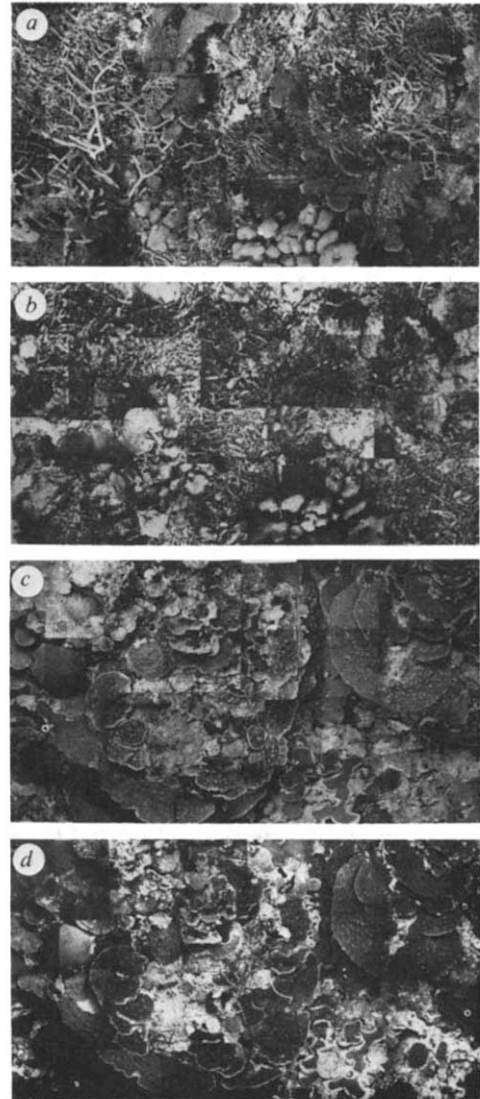


Fig. 1 *a*, Photostation IV at 6.7 m depth, Monitor Reef, Discovery Bay, Jamaica, 28 September 1976; *b*, same station, 14 August 1980, 8 days after Hurricane Allen. *c*, Photostation XII in 32.3 m depth, Monitor Reef, 1 October 1976; *d*, same station, 14 August 1980, post-Hurricane Allen. Photostation montages shown are constructed from 32 individual $0.5 \times 0.5 \text{ m}$ photographic frames that cover a $2 \times 4 \text{ m}$ plot; 8 m^2 is sufficient to define per cent cover, relative abundance, cumulative species number and average number of colonies per m^2 such that a doubling of this sample size would not be expected to change the value of the measured parameter by more than 5% (ref. 34). In comparing *a* and *b*, note the differential removal of the spatially dominant branching *Acropora* species and the creation of a pavement of fragmented coral branches.

Acropora commonness *per se* but from its morphology and concomitant susceptibility to wave damage.

In the deeper reef station (PS XII, 32.2 m depth), our detailed analysis did not reveal any effects of the storm (Fig. 1c, d). In the 4 yr before Hurricane Allen, no apparent relationship existed between the per cent cover of a coral species and the likelihood that it would lose or gain area in a subsequent survey; nor did this pattern change in the immediate post-hurricane survey. Recruitment patterns have not varied from year to year in the deep reef station, but, in contrast to recruitment patterns in shallow water which showed no relationship between loss and recruitment, there is a highly significant (Spearman rank correlation $r_s = +0.55$; $0.01 > P > 0.005$) positive correlation between the loss of colonies and their recruitment into the deeper reef station.

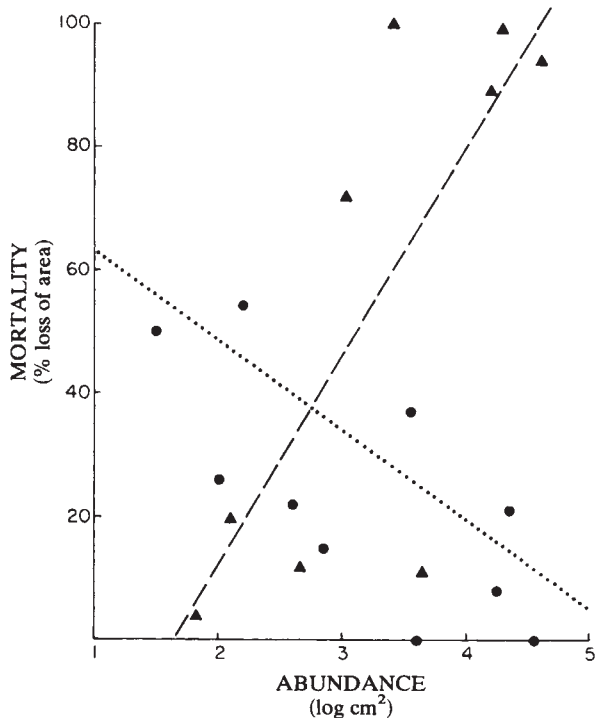


Fig. 2 Loss of existing area by each species in a subsequent survey (●, September 1976 to September 1978, pre-Hurricane Allen; ▲, September 1978 to August 1980, post-Hurricane Allen) is plotted against the abundance of that species in shallow-water photostations II and IV. Photographs are analysed from area covered by each separate colony with a Hewlett-Packard 9815A-9864A digitizer. Mortality was measured as the disappearance of colony area from one survey to the next and includes mortality from physical factors, disease, predation, extra-coelenteric digestion and occlusion from the camera's eye due to the overgrowth of one species by another. We assume overtopping reduced fitness of fully shaded lower individuals and have demonstrated in photostations II and IV that the removal of canopy branches of *Acropora palmata* reveals many dead coral colonies which were living just before being overtopped between 1976 and 1980²³. Mortality was negatively correlated with abundance for the years preceding Hurricane Allen (Spearman rank correlation $r_s = -0.74$; $0.01 > P > 0.005$; $n = 10$). While all species lost some area during the storm, the pre-hurricane pattern of disproportionate loss among the rarer coral species was reversed, with natural selection significantly favouring corals of lesser abundance ($r_s = +0.65$; $0.05 > P > 0.025$; $n = 9$). Trend lines are determined by linear regression. Rank order for adequately sampled coral species (three or more colonies and at least $1.0 \times 10^2 \text{ cm}^2$) for photostations II and IV for both years is: *A. palmata* (PS II), *Acropora prolifera* (PS IV), *A. palmata* (PS IV), *Montastrea annularis* (PS IV), *Acropora cervicornis* (PS IV), *Agaricia agaricites* (PS IV), *A. agaricites* (PS II), *Porites astreoides* (PS II), *M. annularis* (PS II) and *Millepora complanata* (PS IV). The rarest species included in the initial survey (1976-78), *M. complanata*, was too rare to sample adequately in the subsequent survey (1978-80) because it was almost completely overgrown by 1978.

Hurricane Allen has produced a complex biological and morphological rearrangement on the Jamaican reef, in striking contrast to the original organization. Before the storm, shallow water mortality was strongly influenced by biological controls of competition through overgrowth; during the storm, mortality was predominantly controlled by physical factors, with heaviest losses among the commonest, competitively superior (but with respect to the storm, morphologically inferior) species. Hurricane Allen has temporarily eliminated overtopping as a significant agent of mortality in shallow water Jamaican reefs. Although patterns of mortality reversed during the storm, it must be kept in mind that the post-hurricane shallow reef retains slightly less than 10% of the original coral cover, and the predictive model (exemplified by Fig. 2) that diversity will increase until crowding slowly reduces it, remains untested by recovery data. Despite the proven regenerative powers of coral fragments²⁹⁻³², in the period following the hurricane there has been a high mortality among *Acropora* branchlets tagged or photographed immediately after the storm³³. It remains to be demonstrated whether any of these asexually produced pieces will survive to contribute significantly to the regenerating reef.

In deeper water, more equitable competitive abilities among species and slower growth rates could retard competitive exclusion, allowing lower disturbance levels to act successfully as diversifying forces^{2,4,7}. Compensatory processes of recruitment, quantified in the present study, might also promote species richness.

This report is part of a long-term study of changes in community structure. It contrasts those changes occurring under the slow influence of continuously acting or chronic factors with those due to sudden but rare catastrophic disturbances. We are continuing to document the successional dynamics of recovery after this natural experiment so as to quantify better the mechanisms by which patterns in these communities arise, persist or change.

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Evidence for delayed mortality in hurricane-damaged Jamaican staghorn corals

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Severe tropical storms can cause widespread mortality in reef corals^{1,2}. The Caribbean staghorn coral, *Acropora cervicornis*, although dependent on fragmentation for asexual propagation³⁻⁵, is particularly vulnerable to hurricane damage^{6,7}. The most important agents of post-hurricane mortality are assumed to be high wave energy⁶ and change in salinity⁸, factors which typically soon diminish in intensity. We report here that there was substantial delayed tissue and colony death in *A. cervicornis* on a Jamaican reef damaged by Hurricane Allen. This previously undocumented degree of secondary mortality, sustained for 5 months and unrelated to emersion⁹, was over one order of magnitude more severe than that caused by the immediate effects of the storm. The elimination of >98% of the original survivors suggests potentially complex responses to catastrophes, involving disease^{10,11} and predation, which may explain the widely variable rates of reef recovery previously reported¹²⁻¹⁵.

Hurricane Allen passed within 65 km of the reefs of Discovery Bay on 6 August 1980 (refs 7, 16). This storm, the strongest recorded for the Caribbean, generated massive waves (>6–12 m) but little rain⁷. The large mounds of the delicately branching *A. cervicornis*, which had dominated the West Fore Reef between 6 and 20 m depth^{4,17}, were extensively fragmented^{7,16}. To assess the long-term effects, we surveyed all pieces of *A. cervicornis* having living tissue in three haphazardly chosen 1-m² areas of moderate to severe damage at 8 (n = 125) and 14 (n = 129) m depth on Dancing Lady Reef 3–9 days after the storm. These pieces were all detached by the hurricane, the skeletal break often (53%) occurring where there had been no living tissue⁴. Although the once open network of branches was compacted, much of the surviving tissue was not smothered by direct contact with other fragments or the underlying solid reef base. The median number of living or partially living branches per fragment was 3 (range 1–19), and 53% of the fragments had at least one living axial (terminal) polyp (median = 1, range 0–17). The median total length of living tissue in these quadrats was 916 (range 457–1,250) cm m⁻², ~40% of 1977 estimates¹⁸. We individually tagged each living staghorn in the six quadrats and returned at irregular intervals to re-examine them.

Mortality continued at an unexpectedly high rate (Fig. 1). For censuses made within 4 weeks of the storm, there were highly significant differences at both depths (after tagging) between surviving and dead colonies in the lengths (when tagged) of their longest living branches ($P < 0.001$, Mann-Whitney U test), but this difference showed reduced significance by 7 weeks ($P < 0.03$) and was absent by 12 weeks ($P > 0.1$). The relationship between the total number and presence of axial polyps on the longest living branch and survival was not strong ($0.05 > P > 0.01$ at any depth for one census only). Other attributes (depth, branching order¹⁹, number of live branches, orientation of longest living branch and relative height of fragment off bottom) showed no significant relationship with survival. Five months after the storm, we found only four living colonies within the six

quadrats, and median total length of living tissue was reduced, from 916 to 10 (range 0–26) cm m⁻². Thus there had been a nearly 100-fold long-term decrease in living colonies from the original post-storm number. Examination of other areas on Dancing Lady and other Discovery Bay reefs indicated that our quadrats were not atypical.

Two 18-m² transects were established near the shallow and deep sites following the death of most of the originally tagged corals by January 1981. Surveys of these transects showed that, in some areas, tissue mortality still exceeded new growth (indicated by lengthening branches and production of new axial polyps) nearly 1 yr after the hurricane. At 8 m depth there was a 26% reduction in live tissue (from 27 to 20 cm m⁻²) between mid-March and late July, while at 14 m depth there was an increase of 11% (from 26 to 29 cm m⁻²). In both transects the number of living fragments had declined during this interval (from 25 to 17 at 8 m, and from 23 to 17 at 14 m).

There are many possible explanations for this delayed mortality. Fragmentation might (1) facilitate continuing abrasion by the rolling of free fragments¹²; (2) decrease the 'three-dimensionality' of the reef, resulting in smothering or easier access for benthic predators of corals⁴; or (3) increase the susceptibility or exposure of colonies to disease^{10,11}. *A. cervicornis* could be particularly susceptible to these processes because of its growth form, sensitivity to stress (for example, extreme temperature^{20,21} and low salinity⁸), and its apparently high vulnerability to disease¹¹ (perhaps partly a consequence of its tendency to form large stands, hence increasing the potential for epidemics²²). Differential initial mortality of staghorn coral and its predators, competitors²³, prey and pathogens could upset pre-storm relationships, leading to the coral's sharp decline. Some of these mechanisms seem to have had no major effect: we observed little evidence of burial by sediment or continuing mechanical abrasion, even on days with large swells. There was no obvious, long-term increase in competition with algae; species which showed 'blooms' within a month of the hurricane⁷ have since died back.

During the first few months, much of the mortality was probably related to continuing disease and fragmentation stress. Unusual amounts of tissue exfoliation, resembling that termed¹⁰ 'white band disease' were observed in some colonies of *A. cervicornis* for 2 months before the hurricane⁷. This exfoliation continued after the storm, and could not be reliably distinguished from predation (see below) for at least 1 month. Although a relationship between fragmentation and disease has been reported for *A. cervicornis* in Curacao¹¹, we found no persistent relationship between survival and fragment length, orientation or elevation. There is, moreover, some evidence for delayed post-hurricane mortality in species of *Acropora* not showing extensive tissue loss beforehand (*Acropora palmata* in Jamaica⁷ and *Acropora prolifera* in St Croix; R. D. Clarke, personal communication).

By January 1981 mortality was primarily related to the activities of species that were previously important sources of

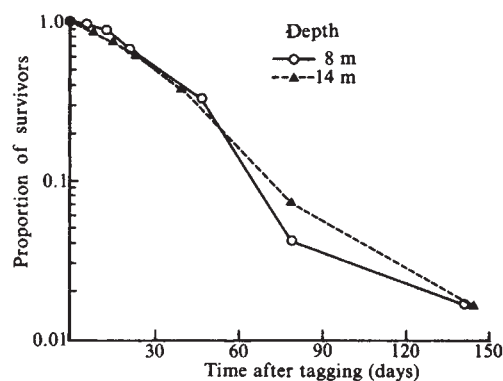


Fig. 1 Proportion of fragments surviving as a function of the time elapsed after tagging. Each fragment had one or more patches of living tissue, and was tagged 3–9 days after the hurricane.

Table 1 Estimates of mean predator densities (per m²) before and after Hurricane Allen

Species	Depth, Location	Density (m ⁻²)	
		(Before hurricane)	(After hurricane)
<i>Diadema antillarum</i>	8 m, DL	13.5 (30)	8.4 (16)
<i>Diadema antillarum</i>	14 m, DL	6.0 (30)	2.6 (25)
<i>Eupomacentrus planifrons</i>	14 m, DL	1.8 (30)	1.3 (25)
<i>Coralliophila abbreviata</i>	9 m, LTS	0.6 (40)	0.3 (25)
<i>Coralliophila abbreviata</i>	14 m, DL	0.6 (40)	0.1 (200)

Reefs surveyed were Dancing Lady (DL) and LTS (reef immediately west of DL); numbers in parentheses indicate numbers of 1-m² areas examined. The only values available for before the hurricane are based on surveys by Kaufman²⁴ (1977; *Diadema*, *Eupomacentrus*) and L. S. Land (unpublished results, 1975; *Coralliophila*), but these seemed to be representative of pre-storm conditions in 1980. 'After' densities were assessed in February–March 1981.

mortality in Discovery Bay. Predation by the snail *Coralliophila abbreviata*^{4,24,27} has been particularly severe in shallow water; 1-m² quadrats along the transect at 8-m depth containing living staghorn and snails in May showed a median decrease by July in total staghorn length of 27.5 cm, whereas quadrats containing coral but no *C. abbreviata* showed a median increase of 2 cm ($P < 0.01$, Mann–Whitney U test). Disturbance by the urchin, *Diadema antillarum*^{24,25}, and the damselfish, *Eupomacentrus planifrons*²⁴, has also contributed to the observed coral deaths. In March, densities of these species were generally lower but within an order of magnitude of pre-storm estimates (Table 1), in contrast to the 100-fold decrease suffered by the staghorn. Thus predation pressure per colony of surviving *A. cervicornis* is now substantially higher.

Several important points emerge from this study. First, future studies of catastrophic damage to reefs (and other ecosystems) should be started as soon as possible after the event. By 5 months, for example, many tagged dead pieces had eroded calices, thus this characteristic¹⁴ could not by then be used to distinguish between pieces dying before and after the storm²⁶. Second, the geographical and temporal scale over which catastrophes occur could critically influence subsequent events. For example, destabilization of relationships between corals and their predators is more likely to occur when the area of coral destruction is great; adult predators of limited mobility, unable to emigrate from decimated regions, will continue to feed in areas where new coral growth can no longer keep pace with predation. The resulting drop in coral abundance will be particularly severe if predators do not succumb rapidly to starvation. Similarly, when substantial coral mortality (for whatever reason) occurs only rarely, the relative abundances of associated reef organisms are more likely to be strongly shifted²⁸. Thus survival probabilities and regeneration rates observed after routine levels of fragmentation (experimental or natural^{4,5,11}) may tell us little of the reef's potential for recovery from unusually extensive damage¹³.

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Carbon-13 isotopic fractionation as a measure of aquatic metabolism

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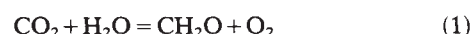
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The fixation of dissolved CO₂ in organic matter during photosynthesis preferentially removes ¹²C from the water, causing the remaining HCO₃⁻ to become enriched in the less abundant isotope, ¹³C. Respiration later releases part of this CO₂ back into the water. Metabolically active aquatic communities thus can generate variations in both the chemical^{1–3} and isotopic^{4–7} compositions of the water surrounding them. We describe here experiments designed to establish the isotopic fractionation of carbon by the metabolism of two common coral reef organisms. We then show that the fractionation coefficient obtained for the metabolism of those organisms is also applicable to a reef community and may be used to estimate the metabolic rate of the community.

In our first experiment, we placed specimens of the common Indo-Pacific reef-building coral, *Pocillopora damicornis*, in a 90-litre aquarium and alternately placed the aquarium in sunlight and darkness during a 6-h period. The aquarium was covered with a sheet of clear plastic (Mylar) to impede gas exchange across the air–water interface. Samples were collected for measurement of pH and alkalinity and calculation of ΣCO₂ (ref. 8), and duplicate samples were processed for carbon and oxygen isotopic analyses⁹. We shall consider here only the carbon data. The isotopic data are reported in the familiar δ notation: $\delta^{13}\text{C} = [(R_{\text{sample}} - R_{\text{std}})/R_{\text{std}}] \times 1,000$ where R is the ratio ¹³C/¹²C and δ¹³C is reported in parts per thousand (‰) relative to PDB. The second experiment was similar, except that we used the common reef-flat red alga, *Acanthophora specifera*, and the incubations occurred over a 24-h period.

In the third experiment, water samples were collected from a coral reef in Kaneohe Bay, Hawaii, over a mixed algal/sand community in water about 3 m deep. Procedures were similar to those used for the two aquarium experiments, except that the water over the reef community was open to gas exchange with the atmosphere. The observed range of ΣCO₂ variation in the field experiment was about 80 μmol l⁻¹ compared with 300 μmol l⁻¹ in the aquarium experiments. Water flow across the reef is typically <5 cm s⁻¹. Therefore samples were collected at one location at selected 1-h intervals over a 24-h period.

Consider the simplified metabolic reaction:



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The progressive utilization of ΣCO_2 as the reaction proceeds from left to right represents photosynthesis and should be described by a change of $\delta^{13}\text{C}$ as a function of decreasing ΣCO_2 . Similarly, respiration (the metabolic reaction proceeding from right to left) should be described by a change of $\delta^{13}\text{C}$ as a function of increasing ΣCO_2 .

Broecker and Oversby¹⁰ demonstrate the use of the Rayleigh distillation equation to describe the progressive change in the isotopic composition of some material, X , as that material is removed from the initial dissolved phase, X_0 according to a single-stage fractionation model:

$$\frac{R_x}{R_{x_0}} = f^{\alpha-1} \quad (2)$$

where R_{x_0} is the starting isotopic ratio, R_x the isotopic ratio in the dissolved phase at any time during reaction, f the proportion of X remaining in the dissolved phase at that time in the reaction and α the fractionation coefficient. Equation (2) can be rewritten in a form that is convenient for numerical computation:

$$\Delta\delta_x \approx \epsilon \ln f \quad (3)$$

where $\epsilon = 1,000(\alpha - 1)$ and $\Delta\delta_x$ represents the change in the $\delta^{13}\text{C}$ of the ΣCO_2 in solution. Thus, $\Delta\delta_x$ versus $\ln f$ should have a slope ϵ . At any point in a Rayleigh distillation, the product material will differ from the remaining material by approximately $\epsilon\%$.

'Reverse distillation' (the release of CO_2 from organic matter to the dissolved phase) may follow the distillation line if the material utilized is released without fractionation. This condition need not apply, although it applies here to the carbon metabolism data.

In the case of calcifying organisms, such as corals, or calcifying communities, such as coral reefs, variations in ΣCO_2 and $\delta^{13}\text{C}$ of ΣCO_2 reflect both calcification and organic metabolism. The $\delta^{13}\text{C}$ of coral reef CaCO_3 is near the $\delta^{13}\text{C}$ of ΣCO_2 in seawater (0 to -5% ; see, for example, ref. 11), whereas the $\delta^{13}\text{C}$ of organic material from coral reefs is about -14 to -18% (ref. 12). Moreover, calcification ordinarily induces CO_2 changes substantially smaller than CO_2 changes from organic carbon metabolism^{6,8}. We conclude that, as calcification is a weakly fractionating reaction, we can compare $\delta^{13}\text{C}$ with calcification-corrected ΣCO_2 as an indicator of organic metabolism (calcification correction is $\Delta \text{total alkalinity}/2$)^{6,8,13,14}.

Figure 1 describes the relationship between $\Delta\delta^{13}\text{C}$ and calcification-corrected ΣCO_2 (expressed as the fraction remaining), as determined by the aquarium experiments. Note that for CO_2 released during respiration, the data are described by the same regression relationship as the distillation, or uptake, curve. For this $\delta^{13}\text{C}$ fractionation, ϵ is -18.6% ($\sigma = 0.7\%$).

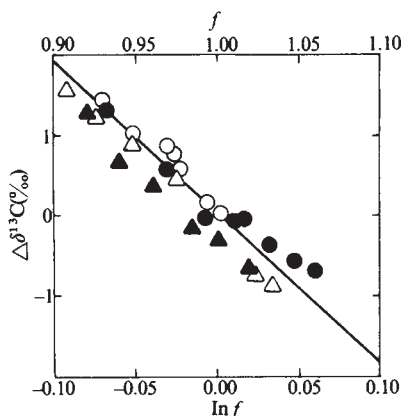


Fig. 1 Relationship between the change in $\delta^{13}\text{C}$ ($\Delta\delta^{13}\text{C}$) and the fraction of calcification-corrected ΣCO_2 remaining $\{[\text{CO}_2 - (\Delta \text{alk}/2)]/\text{CO}_2 \text{ initial}\}$ in laboratory incubations of corals (light, $\circ$; dark $\bullet$) and algae (light, $\triangle$; dark, $\blacktriangle$). The geometric regression line is $\delta^{13}\text{C} = 0.04 - 18.6 \ln f$.

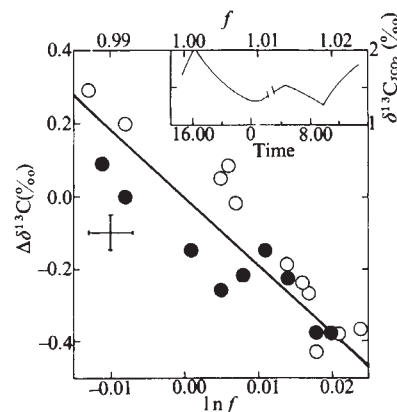


Fig. 2 Relationship between $\Delta\delta^{13}\text{C}$ and the fraction of calcification-corrected ΣCO_2 remaining from field experiment on algal-coral reef community. Also included is the variation of the $\delta^{13}\text{C}$ of the ΣCO_2 as a function of the time of day. The offset between midnight and 5 a.m. represents new water from outside the reef. Note the $5\times$ scale expansion relative to Fig. 1. The cross represents the analytical precision of the measurements. The line through the data is that calculated for the laboratory experiments. $\circ$, Light; $\bullet$, dark.

We offer the following independent estimate of the carbon isotopic fractionation coefficient. During our experiments, the seawater ΣCO_2 had an average $\delta^{13}\text{C}$ of $\sim 2\%$; a fractionation of -19% would result in the tissue having a $\delta^{13}\text{C}$ of about -17% . This is in agreement with observed values of -14 to -18% (ref. 12). $\delta^{13}\text{C}$ can be routinely measured with a precision of $\pm 0.03\%$. The ΣCO_2 content of seawater is about $2,000 \mu\text{mol l}^{-1}$. The precision with which ΣCO_2 changes can be inferred from isotopic changes is calculated from equation (3) to be about $3 \mu\text{mol l}^{-1}$.

Figure 2 shows the data from the field experiment. The geometric regression slope derived from the field data (-18.8) is virtually identical with the laboratory data (-18.6). Daytime $\delta^{13}\text{C}$ increases and night-time $\delta^{13}\text{C}$ decreases average $\sim 0.09\%$ h^{-1} . Using $\epsilon = -19$ and the ΣCO_2 of seawater = $2,000 \mu\text{mol l}^{-1}$, solving equation (3) gives an average rate of change of ΣCO_2 of $-9 \mu\text{mol l}^{-1} \text{h}^{-1}$ in the daytime and $+9 \mu\text{mol l}^{-1} \text{h}^{-1}$ at night. Summing the daytime rate over 12 h and multiplying by 3 m water depth yields a daytime production rate for the entire water column of $3.9 \text{ g C m}^{-2} \text{ day}^{-1}$. A 24-h summation for the night-time rate data yields a respiration rate of 7.8 g C per m^2 per day. Gross production is calculated as half the 24-h respiration plus the daytime production, or 7.8 g C per m^2 per day¹⁵. These figures are close to previous estimates⁶ that coral-reef organic carbon production is 5 – 10 g C per m^2 per day.

We conclude that natural ^{13}C isotopic changes can be used to calculate aquatic metabolic rates. We have measured the fractionation coefficient for this process by inducing relatively large composition changes in laboratory aquaria and then have validated our model with field data. It should be possible to apply this approach to a variety of biological communities.

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Bacillus anthracis on Gruinard Island

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During the Second World War, trials of *Bacillus anthracis* as a potential agent of biological warfare (BW) were carried out on Gruinard Island off the west coast of Scotland (57°56' N, 5°35' W). Small bombs containing a slurry of spores of *B. anthracis* were detonated: most were suspended, about 6 feet above ground, from a gantry but one was dropped from an aircraft. The resulting aerosol clouds of spores were observed to pass through lines of sheep tethered at various distances downwind of the detonation site and after a few days several sheep died of anthrax. The lethal nature of BW weapons used in the open had, therefore, been shown; the unfortunate legacy was an island heavily contaminated with the persistent spores of a virulent microorganism. Soil samples were tested annually from 1948 to 1968 and again in 1972 by Ministry of Defence staff and were found to contain viable spores of *B. anthracis* (unpublished work), although accurate counts were not made. We report here the results of the first full survey of the extent of the contaminated area and the numbers of viable spores of *B. anthracis* present. The survey, carried out in 1979, shows that areas around the gantry remain contaminated to a detectable level but a much wider surrounding zone of undetectable contamination, possibly containing localized high concentrations of spores, could also constitute a hazard.

The whole island was marked out with a grid system (Fig. 1), soil core samples being taken at each marker by pushing a hollow tube (350 mm long × 25 mm internal diameter) into the ground to a maximum depth of ~300 mm. In the area close to the side of the gantry, 10 closely grouped cores were taken and a second similar group was collected ~50 m north west of the gantry in an area which had been traversed by the spore clouds. In the laboratory, the cores were expelled from their tubes into glass jars and shaken with twice their weight of 0.1 M phosphate buffer (pH 7.0) for 3 min on a 300 r.p.m. orbital shaker. The soil-buffer mixtures were heated at 60 °C for 1 h to destroy vegetative microorganisms, thus ensuring that only spores would be cultured. After allowing large particles to settle, 0.2 ml of the supernatant fluid was spread on each of five nutrient agar plates (Oxoid) which were then incubated at 37 °C for 24 h.

The growth of the natural aerobic spore-forming soil microflora was so profuse that it was necessary to dilute the soil extract at least 1 in 2 to distinguish colonies of *B. anthracis*, and consequently the presence of low numbers of *B. anthracis* spores would probably not have been detected. Colonies on nutrient agar which were suspected of being those of *B. anthracis* were isolated in pure culture and subjected to various confirmatory tests, phage sensitivity¹ and the 'string of pearls test'² which is based on the sensitivity of the organism to penicillin. The mouse LD₅₀ of isolates satisfying the confirmatory tests was determined by inoculating spores intraperitoneally in mice. A comparison between the Vollum laboratory strain (LD₅₀, 49 spores; 95% confidence limits 20–91 spores) and isolates from the island (LD₅₀, 115 spores; 95% confidence limits 38–270 spores) indicated that the latter had not lost their virulence for mice. Blood smears stained with polychrome methylene blue invariably showed the presence of the vegetative rods of *B. anthracis*, characteristically dark blue with a pink capsule. *B. anthracis* was detected in 20 out of 153 soil samples. All the positive samples were taken close to the gantry and at 50 m north west of the gantry; the numbers were small (Table 1) and we did not detect *B. anthracis* in any of the samples taken at the grid markers.

To confirm and extend the above technique we developed one that uses a selective medium and will detect as little as 3 spores per g of soil (Table 2). This involves mixing Gruinard soil with twice its weight of distilled water and agitating vigorously in a domestic blender for 5 min. The soil slurry was strained through a double layer of cotton gauze and 0.2-ml volumes were spread undiluted on the medium devised by Kniseley³, which allowed growth of colonies of *B. anthracis* but suppressed growth of the normal soil microflora. Phosphate buffer pH 7.2, water adjusted

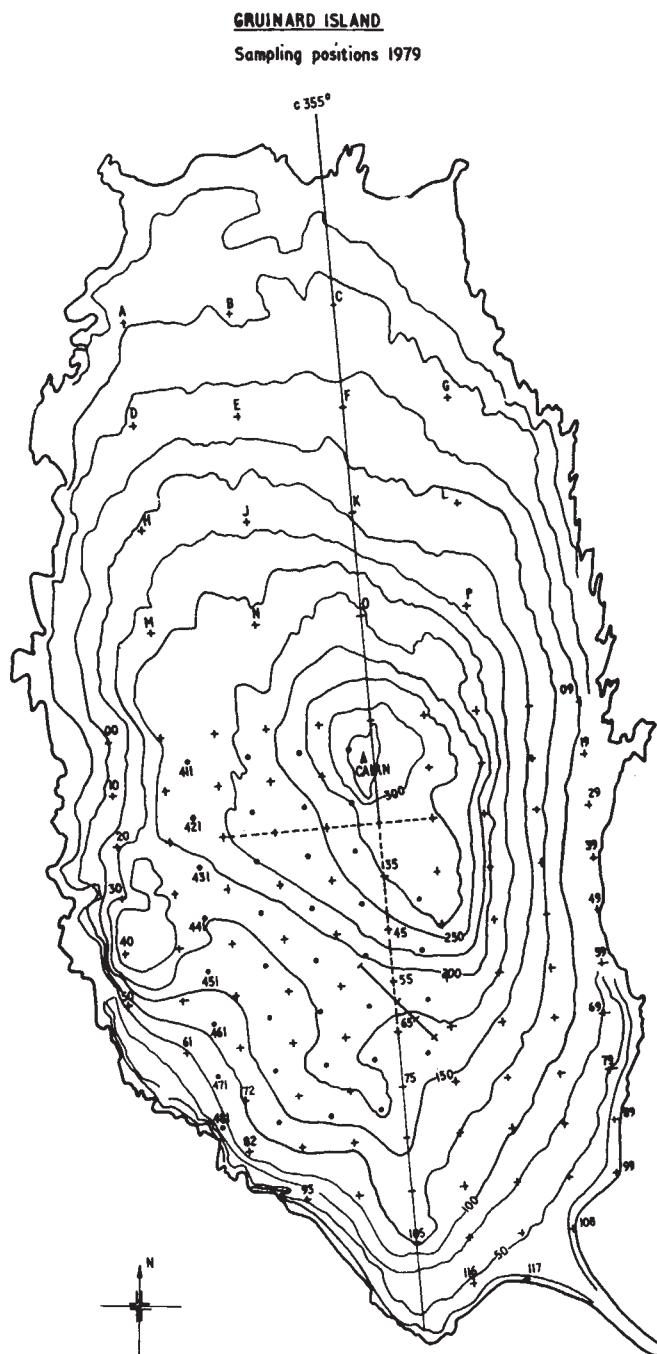


Fig. 1 The northern half of Gruinard Island was subdivided by a 200-m grid represented by the letters A–P, and the southern half by a 100-m grid numbered 00–117. Each cross represents a marker post. The southern grid was further subdivided by placing a marker post represented by ● at the intersections of the diagonals of the 100-m squares and the lines of these posts were numbered 411, 421, etc. The area where BW trials were carried out is indicated by the short line running NW–SE between markers 55 and 65. The gantry location is indicated by the intersect at the SE end of the line and the other intersects represent distances of 50, 100 and 200 yards downwind (scale 1: 10,000).

Table 1 Number of spores of *B. anthracis* detected per g of soil in samples close to the gantry and at 50 yards NW of the gantry

Site	1	2	Core no. 3	4	5
Gantry	312	115	385	270	125
50 yards NW	46	64	144	120	32

Table 2 Distribution of spores of *B. anthracis* with soil depth

Depth (cm)	Spore count per core in each of four cores				Average spore count per g of soil	% At indicated depth
	1	2	3	4		
0-2	270	1,110	810	180	68	43
2-4	18	189	1,704	540	71	43
4-6	99	54	293	33	12	13
6-8	—	30	29	0	2	1
8-14	—	0	0	0	0	0

Cores collected from the gantry area were sliced into sections 2 cm deep. Each slice was assayed individually for its content of spores of *B. anthracis*. — Indicates that the core did not extend to that depth.

to pH values between 5 and 9, and an aqueous solution of 0.1% Tween 80 were also tried as extraction fluids, but no improvements were apparent compared with distilled water. In routine use one viable spore present in a 1-ml sample of soil extract could be successfully cultured. This represents 3 spores per g of soil, equivalent to 3×10^5 spores per m² of ground. Using the new technique, four times more colonies of *B. anthracis* were observed than with nutrient agar, and at low levels of contamination (<12 spores per g) *B. anthracis* was detected by the new technique in samples which otherwise gave negative results.

One of the most important considerations was the distribution of spores with soil depth. Nearly all the viable spores of *B. anthracis* were found in the top 6 cm of soil (Table 2). This superficial distribution may reflect the slow accumulation of humus on the surface of the soil in this habitat which will delay the rate at which spores become buried.

It has been suggested that in suitable conditions spores of *B. anthracis* will germinate and proliferate⁴. The presence of small pools of water, neutral to slightly alkaline in reaction, containing decaying vegetation as a nutrient source, and warm weather might encourage the pathogen to multiply. It is our view that such conditions do not occur on Gruinard Island. The soil is acid (pH 4.2-4.7), the native flora slow to decay and the weather generally cool. We have shown that if soil cores are incubated at 37 or 22 °C the spores remain dormant and that spores prepared in the laboratory will not germinate on culture media prepared from extracts of Gruinard Island soil. Although $\sim 4 \times 10^{14}$ spores were released during the BW trials, the recent surveys have revealed that only a small proportion remain near the surface of the soil. To simulate conditions in which multiplication of these remaining spores might occur, such as the

Table 3 Effect of additions of nutrients on spore counts of *B. anthracis*

Core no.	Nutrient	Incubation temperature (°C)	Spore count per core Initial inoculum	Final
1	Blood	37	5×10^2	3.3×10^9
2	Blood	22	5×10^2	3.5×10^5
3	Faeces	37	5×10^2	1.7×10^3
4	Faeces	22	5×10^2	5.7×10^2
5	None	37	5×10^2	3.9×10^2
6	None	22	5×10^2	6.6×10^2

Cores (~100 g) from an uncontaminated area close to the gantry were inoculated with about 500 spores of *B. anthracis* (Vollum) incubated with the indicated nutrient, 5 ml of calf blood or 5 g of rabbit faecal pellets, and assayed for their content of spores of *B. anthracis* after 7 days' incubation.

nutrient body fluids from a dead animal soaking into the soil, calf blood (5 ml) was added to anthrax-containing soil cores (~50 g) which were then incubated for 7 days at 37 or 22 °C. Addition of blood raised the pH of the soil from 4.5 to 7.2 and after incubation at either temperature these cores showed a large increase in their spore count (Table 3). Other cores supplemented with faecal pellets from rabbits demonstrated a small increase in count only after incubation at 37 °C. It is apparent, therefore, that death of an animal in a contaminated area could provide a favourable growth environment for the proliferation of *B. anthracis*, and a short period of warm weather would be sufficient to initiate germination followed by growth and formation of a high concentration of second generation spores at the surface of the soil. Such a combination of circumstances would probably be rare but could produce localized high concentrations of the organism close to the soil surface: these have not been found.

Thus, we have developed a technique which will detect *B. anthracis* spores in Gruinard Island soil at concentrations as low as 3 spores per g of sample, and further work would enable us to define the area contaminated to this level. Nevertheless, it is likely that an area of undetectable contamination would surround such an area. The technique could also be applicable to the examination of soil samples from other localities.

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Regional accumulation of vegetal pole poly(A)⁺ RNA injected into fertilized *Xenopus* eggs

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Cytoplasmic determinants localized in particular regions of the egg seem to be important in cell determination during early embryogenesis^{1,2}. Although their composition is uncertain, there is evidence that some are partly composed of maternal RNA molecules³⁻⁶. Consistent with this possibility, maternal poly(A)⁺ RNA and certain specific RNA sequences are reported to show an uneven distribution in the cytoplasm of oocytes and developing embryos⁷⁻¹¹, suggesting that maternal RNA sequences may contain signals required to distribute themselves in specific cytoplasmic regions of the egg. We have now tested this idea by analysing the distribution of RNA sequences localized in the vegetal pole cytoplasm of *Xenopus laevis* eggs after microinjection into fertilized zygotes. We report that exogenous vegetal pole poly(A)⁺ RNA accumulates along a concentration gradient from the vegetal hemisphere to the animal hemisphere of the injected egg and provide evidence that the concentration gradient is set up by active migration of the injected vegetal pole poly(A)⁺ RNA into the vegetal hemisphere cytoplasm between fertilization and the first cleavage.

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Ovulated eggs were orientated and sectioned with a cryotome into six regions through planes perpendicular to the animal-vegetal axis. The sections containing the polar regions of the egg were pooled into animal and vegetal pole groups, their RNA was extracted and labelled *in vitro* by RNA ligase-catalysed transfer of ^{32}P -cytidine-3',5'-diphosphate to the 3' termini. The labelled RNA was separated into poly(A)⁺ and poly(A)⁻ fractions and injected into either the animal or the vegetal hemisphere of fertilized eggs ~15–20 min after insemination. The injected embryos were incubated at 18 °C until they reached the proper developmental stage. They were then fixed, processed for histological examination and the distribution of labelled RNA along their animal-vegetal axes was determined by autoradiography.

To ensure that the 3' label was not transferred from the injected RNA to another molecular species, some of the histological sections were treated with enzymes before autoradiography. As shown in Table 1, the number of grains developed in the autoradiographs was not markedly affected by DNase treatment. Grain development was severely decreased,

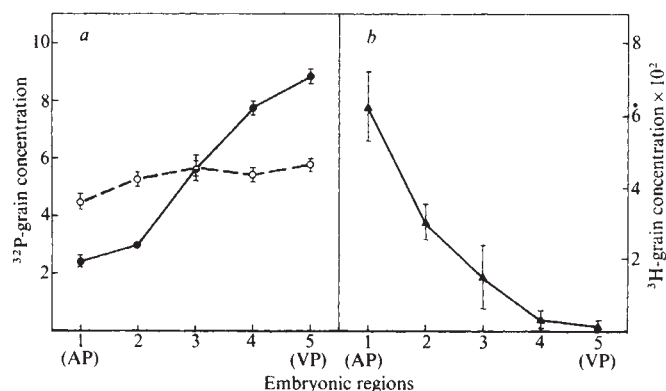


Fig. 1 Grain distribution along the animal-vegetal axis of four-cell embryos after injection of various RNA fractions. Groups of 75–100 deejelled ovulated eggs suspended in a solution containing 20 mM Tris-HCl pH 7.5, 25 mM KCl, 15 mM MgCl₂, 5% sucrose and 0.1% Triton X-100 (Solution A) were oriented animal hemisphere up, frozen in a dry ice-acetone mixture and sectioned with a cryotome into six 200-μm regions perpendicular to the animal-vegetal axis according to the procedure of Moen and Namenwirth¹⁵. The animal and vegetal pole sections were collected, pooled into separate groups and homogenized (five strokes up and down of a Potter-Elvehjem glass homogenizer with a teflon pestle) in 3 vol of Solution A containing 15 μg ml⁻¹ sodium heparin (4 °C). The homogenate was centrifuged at 10,000g for 10 min at 4 °C, the supernatant fraction was collected and adjusted to 0.5% SDS and to 100 mM with Tris-HCl pH 9.0¹⁶. RNA was extracted by shaking the suspension with phenyl/chloroform/isoamyl alcohol (50:50:1). The deproteinized RNA was ethanol precipitated, washed and resuspended (420 μg ml⁻¹) in a solution containing 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1.5 mM ATP, 6.6 mM dithiothreitol, 1.28 Ci ml⁻¹ ^{32}P -cytidine-3',5'-diphosphate (NeN), and ~230 U ml⁻¹ of RNA ligase (EC.6.5.1.3, New England Biolabs). The mixture was incubated at 4 °C for 16 h to transfer the labelled nucleotide to the 3' terminus of the RNA substrate¹⁷. The efficiency of labelling and the integrity of the labelled RNA product was checked by sucrose density gradient centrifugation. RNA labelled to a specific activity of ~3 × 10³ d.p.m. μg⁻¹ was separated into poly(A)⁺ and poly(A)⁻ fractions by oligo(dT)-cellulose chromatography¹⁸, ethanol precipitated, dissolved in the microinjection buffer¹⁹ and injected in 30-nl aliquots into the animal or vegetal hemisphere of fertilized eggs (15–20 min after insemination) by the method of Gurdon¹⁹. The injected volume contained 5–15 ng of poly(A)⁺ RNA or the entire oligo(dT)-cellulose-eluted fraction (poly(A)⁺ RNA) derived from 5–15 ng of total RNA. Taking into account the specific activity of the injected RNA, the number of grains detected in a section cut through a diameter of the one-cell zygote, and the efficiency of autoradiography, we calculate that ~95% of the injected RNA radioactivity entered the egg. Injected embryos were allowed to develop at 18 °C until the four-cell stage, when they were fixed with Bouin's and embedded in paraplast. Sections were cut at 13 μm, extracted with cold 5% trichloroacetic acid, coated with undiluted NTB-2 liquid emulsion (Eastman-Kodak, New York) and exposed for 2 months. The developed slides were stained with Harris haematoxylin and examined by dark-field microscopy to avoid confusing the grains with pigment granules¹⁰. The grains were counted in 400-μm² areas spaced at equidistant intervals (usually ~240 μm, but were varied according to the diameter of the embryo). a, ○, Represent the distribution of the injected vegetal pole poly(A)⁺ RNA and ● the distribution of the injected animal pole poly(A)⁻ RNA; b, ▲ represent the distribution of injected ^3H -polyuridylic acid (NEN). Animal (AP) and vegetal (VP) regions of the embryo are to the left and right respectively.

however, when the sections were treated with RNase in conditions which hydrolyse both RNA and its poly(A) sequence¹². Thus the label remains in RNA after injection.

Figure 1 shows the distribution of grains along the animal-vegetal axis of four cell embryos injected with various RNA fractions after fertilization. When vegetal pole poly(A)⁺ RNA was injected, it appeared to accumulate in a gradient from the vegetal hemisphere to the animal hemisphere with four to five times more grain density in the region nearest the vegetal pole than near the animal pole. Such vegetal to animal hemisphere gradients were observed regardless of whether the vegetal pole poly(A)⁺ RNA was injected into the animal or vegetal hemisphere, suggesting that the entry point on the egg surface was unrelated to the distribution of the exogenous RNA sequences in the cytoplasm. No significant difference in grain distribution was seen along the other primary axes of the embryos. Two control injections were carried out in parallel with the injection of vegetal pole poly(A)⁺ RNA. Labelled polyuridylic acid, whether introduced into the animal or the vegetal hemisphere, became distributed along a steep gradient of opposite polarity to that exhibited by the vegetal pole poly(A)⁺ RNA (Fig. 1), suggesting that the microinjection procedure itself does not dictate the pattern of exogenous RNA distribution. Vegetal pole poly(A)⁻ RNA was also injected into fertilized eggs and, in contrast to the poly(A)⁺ RNA, it showed a relatively homogeneous distribution along the animal-vegetal axis of four-cell embryos (Fig. 1).

The results of the vegetal pole poly(A)⁺ RNA injections do not necessarily imply that this RNA fraction accumulates along a vegetal to animal hemisphere gradient in a specific fashion. Polyadenylic acid accumulates in a similar gradient after injection into fertilized *Xenopus* eggs¹³, and it is conceivable that the poly(A)⁺ RNA passively accumulates in the vegetal hemisphere cytoplasm, possibly because of an adventitious association with yolk platelets. To resolve this issue, we injected labelled poly(A)⁺ RNA from the animal pole region of the ovulated egg into fertilized eggs and analysed its distribution in four-cell embryos. Unlike the vegetal pole poly(A)⁺ RNA, the animal pole poly(A)⁺ RNA did not accumulate primarily in the vegetal hemisphere cytoplasm, but tended to concentrate in the animal hemisphere cytoplasm (Table 2). Others have shown that injected poly(A)⁺ globin mRNA also accumulates along a gradient from the animal to the vegetal hemisphere blastomeres in *Xenopus* embryos¹³. These results suggest that the accumulation of vegetal pole poly(A)⁺ RNA in a vegetal to animal hemisphere gradient is a specific property of the RNA sequences from the vegetal pole region of the egg.

There are three possible explanations for our results: the injected molecules may initially be distributed evenly in the egg and subsequently degraded in the animal hemisphere cytoplasm by localized nucleases; or the injected RNA molecules may be preferentially segregated into the vegetal hemisphere blastomeres by cytoplasmic movements accompanying the second cleavage; or the observed pattern of vegetal pole poly(A)⁺ RNA distribution may be due to selective interaction of the injected RNA with specific recognition sites enriched in the vegetal hemisphere regions of the uncleaved zygote and maintained in the four-cell embryo. If region-specific RNA turnover occurs in

Table 1 The effect of DNase and RNase treatment on grain development in embryos microinjected with labelled RNA

Treatment	Grain concentration ± s.d.	% Of control
None	11.0 ± 1.4	—
DNase	10.0 ± 0.7	90.9
RNase	2.1 ± 1.4	18.2

Sections were treated for 16 h with 50 μg ml⁻¹ bovine pancreatic DNase I (EC 3.1.4.5) dissolved in 100 mM Tris-HCl pH 7.5, 3 mM MgCl₂ or 50 μg ml⁻¹ bovine pancreatic RNase A (EC 3.1.4.22) in 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 1 mM MgCl₂ (refs 8, 12) at 37 °C. Grain counts were made in 400-μm² regions in the central areas of four cell embryos.

Table 2 Grain distribution in the animal and vegetal hemispheres of developing embryos injected with poly(A)⁺ RNA

RNA source	Developmental stage	Sample size	Grain concentration $\pm$ s.d.		Vegetal/animal ratio
			Animal	Vegetal	
Animal pole	4 cell	3	18.0 $\pm$ 1.7	9.3 $\pm$ 3.0	0.5
Vegetal pole	1 cell	4	20.6 $\pm$ 2.0	62.4 $\pm$ 2.4	3.0
Vegetal pole	4 cell	6	17.1 $\pm$ 1.3	43.8 $\pm$ 1.3	2.6
Vegetal pole	Blastula	8	14.6 $\pm$ 3.2	23.7 $\pm$ 4.3	1.6

Animal and vegetal pole poly(A)⁺ RNA was derived from different eggs. In the vegetal pole poly(A)⁺ RNA experiment, clutches of inseminated eggs were injected with equal amounts of RNA, allowed to develop for the desired time at 18 °C, fixed and processed for histology and autoradiography. Grains were counted in a 400- μ m² area in the centre of each hemisphere.

the animal hemisphere cytoplasm, we might expect the distribution of injected vegetal pole poly(A)⁺ RNA between the animal and the vegetal hemisphere regions to be more uniform before the four-cell stage. This possibility was tested by injecting equal amounts of the vegetal pole poly(A)⁺ RNA into a clutch of fertilized eggs and examining its distribution in embryos killed at various times during early embryogenesis. The accumulation of vegetal pole poly(A)⁺ RNA in the vegetal hemisphere was more pronounced in the one cell zygote than in the four-cell embryo or the blastula (Table 2), suggesting that the injected RNA is gradually degraded or modified in the vegetal rather than the animal blastomeres during early embryogenesis, perhaps in concert with the relatively high rate of protein synthesis known to occur in the vegetal cells¹⁴. These results do not support a role for selective degradation of the vegetal hemisphere accumulation of injected vegetal pole poly(A)⁺ RNA. The unequal distribution of vegetal pole poly(A)⁺ RNA as early as 20–30 min after injection, when the embryo is still a single cell (Table 2), is also inconsistent with differential segregation to the vegetal blastomeres during the second cleavage.

The formation of a vegetal to animal pole gradient in fertilized zygotes by poly(A)⁺ RNA from the vegetal pole of unfertilized *Xenopus* eggs is most simply explained by active migration. Although the possibility that some of the labelled termini of the injected poly(A)⁺ RNA are exchanged with endogenous poly(A)⁺ RNA cannot be excluded, it is highly unlikely that this alone could lead to the observed patterns of labelled RNA.

Our results suggest that vegetal pole poly(A)⁺ RNA molecules may recognize specific cytoplasmic binding sites which are distributed along a concentration gradient from the vegetal to the animal pole of the egg. These sites could include cytoskeletal elements, membrane systems, specific types of yolk particles or other cytoplasmic organelles. It is not known whether information in the RNA sequence or in endogenous protein that may form complexes with the injected RNA molecules is responsible for this interaction. The ability of maternal RNA molecules or their native ribonucleoprotein complexes to recognize specific regions of the egg is consistent with their being cytoplasmic determinants.

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Sodium and potassium channels in demyelinated and remyelinated mammalian nerve

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Demyelination of peripheral axons initially causes failure of action potential conduction, probably because the internodal membrane lacks sodium channels^{1,2}. However, Bostock and Sears³ showed that 3–14 days after demyelination of rat nerve with diphtheria toxin, some axons develop regenerative inward currents in the internodal membrane permitting continuous (non-saltatory) conduction. In nerves that have remyelinated or regenerated, the number of nodes per unit length of fibre characteristically increases greatly^{4,5}. As the internodal membrane does not normally possess sodium channels^{1,2}, both the earlier appearance of continuous conduction in the demyelinated nerve and the later presence of extra nodes in the remyelinated nerve require either a laying down of new sodium channels or a redistribution of the sodium channels in the original nodes. The present experiments examine the changes in the total number of sodium channels, measured by saxitoxin-binding capacity¹, that occur in rabbit sciatic nerves which have been demyelinated *in vivo* with lysolecithin^{2,5} and then allowed to remyelinate. The results provide no evidence for the formation of new sodium channels during the early stage, when continuous conduction may develop, but show clearly that new channels are formed during remyelination.

Sciatic nerves of rabbits under methoxyflurane anaesthesia were injected on one side in the mid-thigh (20–30 mm distal to the sciatic notch) with lysolecithin (40 μ l of a 1% solution under the sheath) and allowed to recover. Reflex function on the affected side was markedly depressed for the first 1–2 weeks, but thereafter recovered. At various times afterwards (1–200 days, see Fig. 1 legend) the rabbits were killed by injection of air into an ear vein, and from each animal both the lysolecithin-treated and control nerves were removed and desheathed. The sodium channel density in 25–30-mm lengths corresponding to the injected region was determined in both the treated and control homogenized nerves from measurement of the saxitoxin-binding capacity¹. The specific activity of the saxitoxin was initially 7 d.p.m. fmol⁻¹, and its radiochemical purity 68%. The mean uptake for the series, 8.4 fmol per mg wet weight, calculated on the basis of the initial purity, is about half that described previously¹, because over the several months of these experiments the specific activity declined substantially through back-exchange of the label into the water of the solution¹. Errors from this source, however, were avoided by expressing the uptake by remyelinated nerve relative to that of the control nerve determined at the same time and in identical conditions. Uptakes were usually determined from a labelled saxitoxin concentration of 5 nM, about four times the value of the equilibrium dissociation constant (K) for saxitoxin in this homogenized nerve¹. Some experiments, done with 20 nM saxitoxin, gave uptakes about the same as with 5 nM for both normal and lysolecithin-treated nerves, confirming that any change in the value of K would not have significantly affected the results.

Histological examination of two nerves at 7 weeks indicated that a large proportion (~70%) of the fibres had remyelinated (and so presumably had originally been demyelinated), as

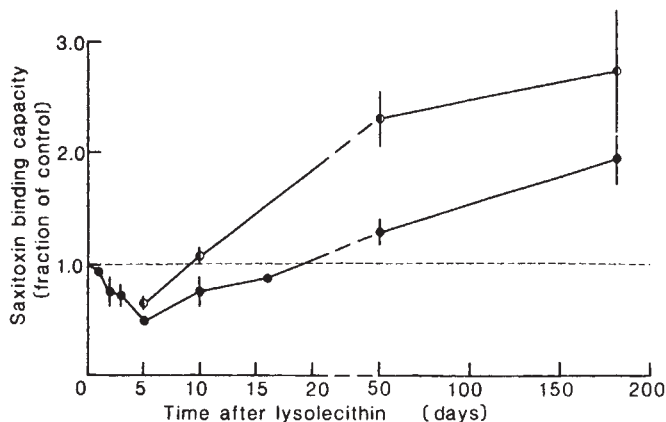


Fig. 1 The uptake of labelled saxitoxin per unit weight (●) and per unit length (◐) of homogenized desheathed rabbit sciatic nerve at various times after injection of 40 μ l lysolecithin (10 mg ml⁻¹) under the sheath *in vivo* (expressed as a fraction of the uptake by the corresponding portion of the contralateral untreated sciatic nerve). The bars represent $\pm$ 1 s.e. (and when absent are hidden by the symbol) and the numbers of experiments at 1, 2, 3, 5, 10, 16, 50 and 180 days were 2, 3, 2, 6, 11, 5, 13 and 3, respectively.

judged by the observation of single axons, each within its own Schwann cell tube, that were smaller in diameter and more thinly ensheathed with myelin than surrounding normal fibres and those in control nerves; in six nerves examined after 7–10 days there were clear signs of demyelination but little or none of degeneration. It would have been interesting to determine the total number of nodes in the tissue at these times. However, this formidable task was not undertaken, and, unfortunately, no such information is available in the literature. In regenerated rabbit nerve, however, where short internodes are found (at least up to 1 yr after injury), Cragg and Thomas⁴ have shown that the average number of nodes per unit length increases 1.4 times for a 10- μ m diameter fibre, and almost doubles for the largest (21–22 μ m diameter) fibres. Remyelination is known to lead to a similar decrease in internodal length⁶ and so, presumably, to a corresponding increase in the number of nodes per unit length.

Initially, the saxitoxin-binding capacity of the lysolecithin-treated nerves (expressed per unit weight of desheathed nerve) decreased relative to that of the contralateral controls, but after 2–6 months the binding capacity increased above its control value (Fig. 1). However, the results as expressed in Fig. 1, comparing the binding per unit weight, are distorted by the connective tissue hyperplasia and oedema that follow intraneural injections; and much more significant effects are seen when comparison is made on the basis of binding per unit length as was done in many of the experiments. For example, 5 days after the lysolecithin injection, the weight per unit length of the injected nerve was $42 \pm 5\%$ greater than in its contralateral control. Thus, expressed per unit weight of nerve, the saxitoxin-binding capacity at this time had fallen to 0.48 ± 0.04 ($n = 6$), whereas, expressed per unit length of nerve, the fall was only to 0.65 ± 0.06 . Similarly, when saline instead of lysolecithin was injected, after 5 days the saxitoxin-binding capacity expressed per unit weight fell to 0.77 ± 0.10 ($n = 4$) whereas, expressed per unit length, it remained roughly constant at 0.96 ± 0.10 . However, ligation of a normal nerve ~ 1 cm distal to the sciatic notch, which involves less surgical interference in the region of the more distal usual injection site, led after 5 days to a fall in channel density in the latter portion of the degenerating nerve that was about the same whether expressed per unit weight or per unit length (to final values of 0.31 ± 0.05 , $n = 4$, and 0.33 ± 0.07 , respectively). Histological examination of two saline-injected nerves after 5 days showed no grossly abnormal morphology of the myelinated fibres.

The early decrease in binding is difficult to interpret without quantitative information about the amount of fibre degeneration caused by the lysolecithin injection, which the experiments on ligated nerves showed would lead to a rapid decrease in toxin binding. Histological examination showed that little degeneration occurred. Because, in principle, the amount of degeneration might be assessed by measuring binding in distal segments of the nerve sufficiently far down to escape any 'spill-over' demyelination, saxitoxin binding was determined in many experiments in regions both proximal and distal to the injected region. In the region proximal to the lesion in 26 nerves examined 5 days–2 months after injection, the weight per unit length on the injected side and the saxitoxin uptake per unit weight were not significantly different from their control values (1.02 ± 0.05 and 1.04 ± 0.10 , respectively). However, in the distal segment the corresponding values were 1.30 ± 0.09 and 0.95 ± 0.10 ($n = 17$), respectively, showing that some spill-over of the effect distally did indeed occur. Even when the portion of the nerve between the knee and the ankle was examined—a region 40–80 mm from the usual site of injection—there was still an increased binding of saxitoxin (per unit length) of $47 \pm 13\%$ (compared with a value of $79 \pm 26\%$ around the injection site) in five nerves that had been injected 7 weeks previously. The morphological basis for this increase remains unclear.

In nerves examined about 2 months after lysolecithin injection, when remyelination was well under way (although not complete because the myelin sheaths were still relatively thin), the weight per unit length was observed to be $65 \pm 8\%$ greater than in the control. The value of 1.30 ± 0.13 for the channel density of remyelinated nerves shown in Fig. 1 for this time thus corresponds to a value for the sodium channel density per unit length of remyelinated nerve of 2.31 ± 0.24 ($n = 13$) that of the control. These experiments, therefore, do not support the idea that the source of sodium channels for the extra number of nodes that appear on remyelination is derived from the population of sodium channels in the original nodes. Rather, there is an increased laying down of extra sodium channels in the membrane, consistent with the increased number of nodes, as if the total number of channels per node remains relatively constant.

In some experiments the nerves were mounted in a capillary chamber containing a series of annular platinum electrodes for electrical recording of the monophasic compound action potential, and exposed for 10–15 min to the potassium channel blocking agent 4-aminopyridine. In frog sciatic nerve such treatment leads to a marked increase in the duration of the compound action potential because the blockage of the repolarizing potassium current leads to a broadening of the individual action potentials in each individual fibre (Fig. 2a). However, in normal rabbit sciatic nerve, as already observed in normal rat nerve⁷, there is at most only a slight broadening of the compound action potential (Fig. 2c, e), presumably reflecting the normal absence of potassium currents^{8,9} in all but a small fraction of the fibres. However, after treatment with lysolecithin, rabbit sciatic nerves acquire a distinct sensitivity to 4-aminopyridine (Fig. 2b, d, f). In five experiments at 7 weeks and three at 6 months the average broadening at half-amplitude, which was $15.6 \pm 3.5\%$ and $29.5 \pm 5.1\%$, respectively, was significantly larger than the broadening of $3.5 \pm 1.6\%$ ($n = 12$) of control nerves ($P < 0.001$). In four experiments at 10 days the broadening of $7.8 \pm 3.5\%$ was not significant ($P > 0.2$).

As illustrated in Fig. 2, the compound action potential in the lysolecithin-treated nerve was consistently wider than in the contralateral control; for example, at 7 weeks the action potential was $47.4 \pm 17.8\%$ broader and conducted $27.2 \pm 11.0\%$ ($n = 5$) more slowly. This indicates that the electrophysiological characteristics of the remyelinated axons differ from those of the normal nerve; however, these differences have not been analysed in more detail.

These findings indicate that not only do new sodium channels appear in the remyelinated nodes but also that in the remyelinated nerve, unlike normal nerve (at least 7 weeks–6 months

after demyelination), potassium channels carry part of the current during the action potential. Whether or not these potassium channels are present in newly formed nodes remains unclear, for Sherratt *et al.*⁷ have shown that some rat nodes of Ranvier show signs of paranodal demyelination a few days after diphtheria toxin and are sensitive to 4-aminopyridine. Therefore, one explanation for the sensitivity to 4-aminopyridine in the present experiments would be that the lyssolecithin had initially caused a paranodal demyelination of some existing nodes, thus exposing pre-existing paranodal potassium channels, and that it was the 4-aminopyridine sensitivity of persisting damaged original

nodes, rather than new nodes, that was observed in the present experiments. Note, however, that for this to account for the results of Fig. 2, any paranodal demyelination existing 7 weeks—6 months after the original exposure to lyssolecithin would have to have persisted longer than is generally the case after lyssolecithin in other mammals^{10–12}.

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Receptor binding of somatostatin-28 is tissue specific

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Although somatostatin was originally identified in the hypothalamus as a tetradecapeptide (SRIF or S-14)¹, subsequent studies have revealed that tissue somatostatin is heterogeneous comprising in addition to S-14 two larger forms with molecular weights (M_s) of 10,000–15,000 and 3,000 (refs 2–7). Recently a 28-amino acid peptide (somatostatin-28, S-28)—a 14-amino acid N-terminal extension of S-14—has been isolated from mammalian gut and hypothalamus^{8–10}. Synthetic S-28 (M_s 3,160) has been shown to correspond to the 3,000- M_s SLI species found in most tissues⁵ and to exhibit greater potency than S-14 for inhibiting both endocrine (growth hormone, insulin, glucagon)^{11,12} and exocrine (pancreatic enzymes and bicarbonate) secretion¹³. It is not clear whether S-28 exerts similar effects to S-14 on central nervous system functions¹⁴ nor whether the S-14-like effects of S-28 are mediated through its conversion to S-14 or through direct action on the recently characterized S-14 receptors of rat brain and pituitary membranes^{15,16}. We suggest here that the greater potency of S-28 for inhibiting growth hormone secretion is because it binds to pituitary S-14 receptors with 3.2-fold higher affinity than does S-14. Furthermore in the central nervous system, S-28 has a lower affinity than S-14 for the receptors, indicating that it is less potent than S-14 in regulating brain functions. Receptor binding of S-28 in both pituitary and brain occurred directly without significant conversion to S-14, suggesting that S-28 is a true S-14 receptor agonist but possesses distinct tissue specificities.

Membrane fractions enriched in somatostatin receptors were prepared from fresh cerebrocortical, hypothalamic and pituitary tissue obtained from adult male Sprague–Dawley rats using techniques reported previously^{15–18}. Tyr¹¹-SRIF was radioiodinated to high specific activity⁷ and used as the radioligand. Figure 1 depicts the specific binding of ¹²⁵I-Tyr¹¹-SRIF to S-14 receptors in the pituitary, hypothalamus and cerebral

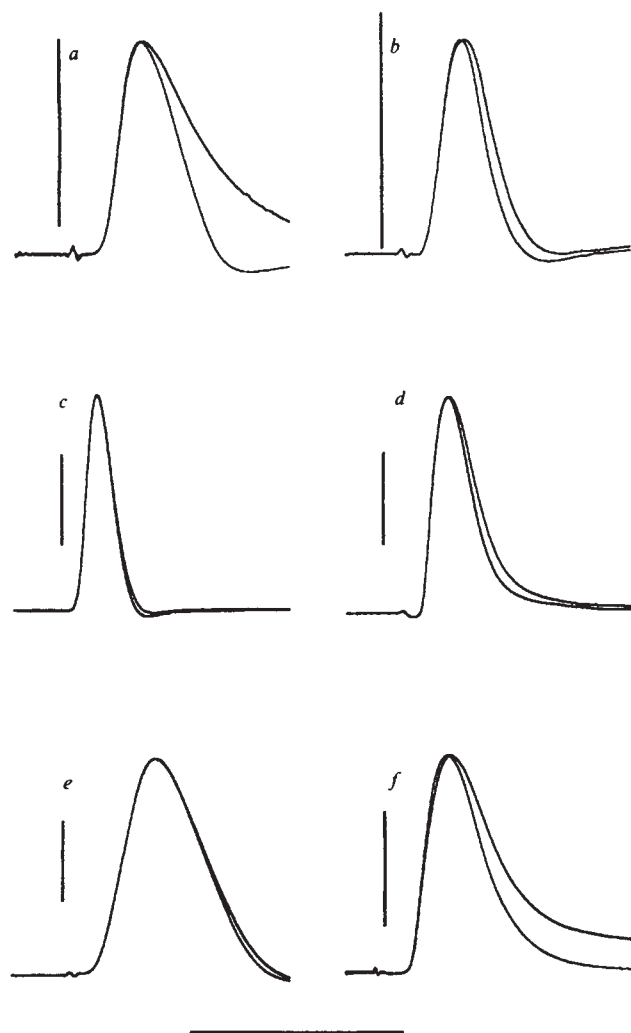
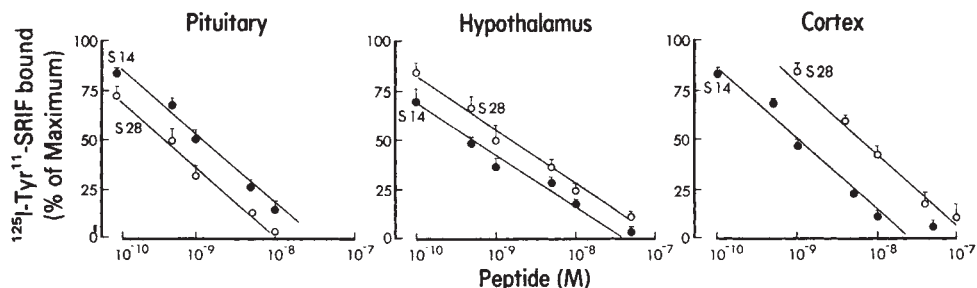


Fig. 2 The effect of 4-aminopyridine on the compound action potential of frog (a) and rabbit (b–f) desheathed sciatic nerves. Each trace consists of a record taken after about 10 min exposure to 4-aminopyridine (200–2,000 μ M) superimposed on the control record before exposure; in all pairs the action potential with the longer duration occurs in the treated nerve. Record a is from a frog nerve at 20°C. Records c and d are from the right and left sciatic nerves, at 37°C, of a 28-week-old rabbit, whose left sciatic nerve had been injected 7 weeks previously with 40 μ l lyssolecithin. Records e and f are the corresponding control and treated nerves, at 20°C, from a 12-month-old rabbit whose left sciatic nerve had been injected with 40 μ l lyssolecithin 6 months previously. Record b shows another example of the broadening by 4-aminopyridine of the compound action potential, at 37°C, of the sciatic nerve of a 28-week-old rabbit injected 2 months previously with lyssolecithin. The vertical bar in each record represents 1 mV for the control record; the record in 4-aminopyridine has sometimes been scaled downwards slightly so that its amplitude is the same as that of the control to allow the shapes of the action potential to be more easily compared. The horizontal bar represents 1 ms for panels a–e and 2 ms for panel f.

Fig. 1 Inhibition by S-28 and S-14 of the specific binding of ^{125}I -Tyr 11 -SRIF to membrane S-14 receptors in the pituitary, hypothalamus and cerebral cortex. Equilibrium binding studies were carried out as described previously¹⁵⁻¹⁸ by incubating 50 μg membrane protein and 5×10^{-11} M ^{125}I -Tyr 11 -SRIF at 30 °C (40 min for brain membranes and 20 min for pituitary membranes) in 50 mM HEPES-KOH buffer, pH 7.5, containing bovine serum albumin (BSA, 10 mg ml $^{-1}$), MgCl $_2$ (5 mM), Trasylol (200 KIU ml $^{-1}$), bacitracin (0.02 μg ml $^{-1}$) and phenylmethylsulphonyl fluoride (0.02 μg ml $^{-1}$). Receptor-bound radioligand was separated by centrifugation of the incubation mixture, and was washed in HEPES-KOH buffer containing BSA (10 mg ml $^{-1}$) and quantified in a γ spectrometer. The specific binding at each concentration was calculated by subtracting the nonspecific binding observed at high concentration of S-14 (1×10^{-7} M) from the total binding. To check the parallelism between inhibition curves of the two peptides, the residual variances of the two curves were tested for homogeneity by an *f*-test and the lines tested for parallelism by a *t*-test, both with the aid of the computer program of Faden and Rodbard²³.



cortex. Both S-14 and S-28 produced dose-dependent inhibition of radioligand binding over a concentration range of 10^{-10} – 10^{-7} M. In each tissue, displacement curves obtained with the two peptides were parallel. The potency of S-28 relative to S-14 was calculated from the concentrations of the two peptides required to inhibit ^{125}I -Tyr 11 -SRIF binding to the membrane receptors by 50%, as shown in Table 1. S-28 exhibited 3.2-fold greater affinity for binding to S-14 receptors in the pituitary, whereas in the hypothalamus and cortex, it exhibited three- and sixfold lower affinities respectively. To determine whether S-28 is converted to S-14 during incubation with the membrane receptors, we analysed the S-28-containing reaction mixtures at equilibrium by Sephadex G-25 (superfine) gel chromatography and radioimmunoassay for somatostatin using an antibody (R149)² reacting 100% with S-28 and S-14. In these conditions, >99% of the immunoreactivity emerging from the columns co-eluted with synthetic S-28, showing that S-28 is not converted to S-14 during incubation with membrane receptors.

These data suggest that the higher potency of S-28 for *in vitro* growth hormone inhibition is due to its greater affinity for binding to pituitary S-14 receptors, as the receptor-binding affinity of the peptide (Table 1) correlates well with its reported 3–14-fold higher potency for growth hormone suppression from cultured rat anterior pituitary cells¹¹. Although S-14 is known to exert characteristic effects in the central nervous system¹⁴, the effects of S-28 on brain have not yet been characterized. Based on our brain receptor binding data, however, S-28 would be expected to show reduced S-14-like biological activity in brain. Because S-28 can be enzymatically cleaved to S-14 (ref. 19), and as breakdown of S-28 to the tetradecapeptide form could influence the comparison of the potency of S-28 relative to S-14, it was necessary to determine the extent of the conversion during our incubation studies. Our finding that S-28 remains intact in the experimental binding conditions indicates that this molecule possesses its own intrinsic receptor binding properties.

What is the physiological significance of S-28? The present data clearly suggest that it is not simply a superactive form of

S-14. As S-28 (or equivalent immunoreactivity) is present in high concentrations in the hypothalamus (approximately one-third of the amount of S-14)²⁰ and seems to be released into the hypophyseal portal circulation²¹, it (together with S-14) is likely to be a regulator of pituitary growth hormone secretion. Likewise, the predominance of the S-28 form of somatostatin in some areas of the intestine⁵ suggests specific regulatory roles for this peptide in this organ. Whether the effects of S-28 are a manifestation of its interaction with S-14 receptors alone or whether there are specific S-28 receptors distinct from those of S-14 remains to be established. The reduced ability of S-28 to compete with ^{125}I -Tyr 11 -SRIF for binding to brain S-14 receptors observed here, together with the recent finding that S-28 competes better with a radioiodinated S-28 analogue (^{125}I -[Leu⁸, D-Trp²², Tyr²⁵]S-28) for binding to brain membranes²² than does S-14, provide evidence for the existence of separate receptors for S-14 and S-28 at least in brain. In any case, our observation that S-28 exhibits receptor-binding activities distinct from those of S-14, the reported differences in the biological actions of the two peptides and the high concentrations of S-28 in some tissues suggest that this molecule not only serves as a precursor of S-14 but also possesses independent physiological functions.

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Table 1 Concentrations (IC_{50}) of S-14 and S-28 required for 50% inhibition of ^{125}I -Tyr 11 -SRIF binding

Tissue	IC_{50} (nM)		Relative potency of S-28*
	S-14	S-28	
Cortex	0.9 $\pm$ 0.12	6.2 $\pm$ 0.11	0.15 (0.07–0.2)
Hypothalamus	0.5 $\pm$ 0.15	1.5 $\pm$ 0.03	0.33 (0.26–0.61)
Pituitary	1.25 $\pm$ 0.22	0.41 $\pm$ 0.014	3.15 (1.98–10.9)

IC_{50} values are mean $\pm$ s.e.m.; *n* = 6 experiments in duplicate.

* Values represent the mean and range of potencies of S-28 (compared with that of S-14 taken as 1) for the six experiments.

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Soluble factor activation of human B lymphocytes

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Medium conditioned by lectin (and/or antigen)-stimulated human peripheral blood lymphocytes has been shown to contain factors, termed interleukin 1 (IL-1) and interleukin 2 (IL-2)^{1,2}, which augment and maintain human T-cell proliferation³⁻⁶, respectively. Interleukin 2 (T-cell growth factor, TCGF) requires the presence of a macrophage, or its monokine product, IL-1, for its production by T cells⁷⁻⁹. It has been shown that multiple signals are required for stimulating both T- and B-cell proliferation⁸⁻¹⁰ but the factors necessary for optimal B-cell mitogenesis have not been rigorously defined. As both B- and T-cell stimulatory agents are present in lectin-stimulated peripheral blood lymphocyte-conditioned media, we studied the effects of the interleukin factors on human B cells. Human peripheral blood B lymphocytes and pokeweed mitogen (PWM)-stimulated B-cell blasts were evaluated for their proliferative response to soluble growth factors, which were obtained from either lipopolysaccharide or phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells. We show here that factors from lipopolysaccharide-stimulated conditioned medium were unable to stimulate initial B-cell proliferation. However, factors from PHA-stimulated conditioned medium stimulated ³H-thymidine (TdR) incorporation in both B cells and lectin-activated T cells. This stimulation could be maintained for multiple re-exposures to the growth factors, which suggests that a soluble factor present in medium conditioned by lectin-stimulated peripheral blood mononuclear cells may modulate B-cell proliferation in a manner analogous to T-cell proliferation.

Human interleukin 1 was purified from mononuclear cell-conditioned media by diafiltration, ultrafiltration and isoelectric focusing (pH 6.8–7.2)¹¹. This material was co-mitogenic for human thymocytes and human T cells at a concentration <1 ng per 25 µl and could not support the continuous growth of cultured T cells¹². Interleukin 2 preparations, containing both T- and B-cell growth factors (see below), were purified from peripheral blood lymphocyte-conditioned media by ammonium sulphate precipitation (50–75% precipitate), DEAE-Sephadex chromatography (0.07 M NaCl elution fraction) and Ultrogel filtration (~25,000-molecular weight (M_r) fraction). This material, at all stages of purification, was capable of stimulating ³H-TdR incorporation in 7-day-old activated T cells and capable of maintaining long-term T-cell growth¹³.

Table 1 illustrates the ability of the soluble factors contained in interleukin preparations to stimulate B-cell ³H-TdR incorporation in the presence of fetal calf serum (FCS). In experiment 1, B cells were purified from peripheral blood by negative selection; they were isolated from those cells remaining after a double adherence protocol and E-rosette depletion. These cells were ≥88% immunoglobulin-positive with the remaining cells composed of null cells, polymorphs and a small percentage of nonspecific esterase-positive cells. The B cells in experiment 2 were further purified by passage over a Sephadex G10 column¹⁴. These cells were >95% surface immunoglobulin-positive (SIg⁺) and contained less than 1% T cells as determined by reactivity to anti-Leu-1 monoclonal antibody. As expected, PWM stimulation of a quiescent B-cell population

Table 1 Effect of soluble factors in interleukin preparations on purified human B cells

	³ H-TdR incorporation (c.p.m.)	
	Expt 1	Expt 2
B cells	1,335	3,650
B cells + PWM	8,228	4,034
B cells + (irradiated) T cells + MØ + PWM*	19,567	23,091
B cells + IL-1†	2,690	ND
B cells + IL-2	40,176‡	38,931§

Cells were cultured in microtitre plates in a final volume of 0.2 ml. Total numbers of B cells added were 0.2×10^6 , T cells (where added) were 0.2×10^6 and MØ were 0.04×10^6 (where added). The cells were grown for 48–72 h then labelled with 1 µCi ³H-TdR for the final 24 h. Standard errors of the mean were routinely <7%. In experiment 1, the B cells were prepared from peripheral venous blood by negative selection. E rosette-positive cells were removed on Ficoll gradients and the nonspecific esterase-positive cells removed by several adherence steps on Petri dishes. After these procedures the cell population was 88% SIg⁺ by immunofluorescence and contained <1% E rosette-positive cells. In experiment 2, peripheral blood B lymphocytes purified as described for experiment 1 were further processed by passage over a G10-Sephadex column. After this step, the recovered population contained 95% SIg⁺ cells and <1% T cells, as determined by E-rosetting and using anti-Leu-1 monoclonal antibody specific for human T cells. ND, not determined.

* PWM was used at a final dilution of 10 µl per ml culture volume (Gibco). T cells were prepared from peripheral venous blood from the nonadherent, E rosette-positive, nylon wool-fractionated population as previously described²⁷. The T cells were irradiated (1,500–2,000 rad) before co-cultivation with the B cells. After irradiation these T cells failed to incorporate significant ³H-TdR in response to lectin exposure.

† In this experiment, IL-1 was used at a 1:100 final dilution. Further experiments using multiple dilutions gave the same results.

‡ The IL-2 preparation (containing both B- and T-cell stimulatory factors) was processed by selective ammonium sulphate precipitation followed by DEAE-Sephadex chromatography, and was used at a final dilution of 1:10. To confirm that residual contaminating T cells (unexposed to lectin) in the B-cell population would not respond to DEAE-chromatographed conditioned medium alone (that is, the IL-2 preparation was free of residual lectin), we tested the preparation on quiescent peripheral blood lymphocytes in comparison with long-term cultured polyclonally activated T cells. ³H-TdR incorporation of long-term cultured lines, in the presence of multiple dilutions of IL-2, was $16,924 \pm 2,537$ c.p.m. at 1:4 dilution and 214 ± 29 at 1:128 dilution. This is in striking contrast to peripheral blood lymphocytes, which showed ³H-TdR incorporation of only 73 and 20 c.p.m. at 1:4 and 1:128 dilution, respectively.

§ The IL-2 preparation (containing both B- and T-cell stimulatory factors) was processed as in experiment 1 but was further fractionated by gel filtration on a Ultrogel AcA54 column. That activity eluting at M_r 24,000 was tested in experiment 2, at a final dilution of 1:20.

Table 2 Evaluation of T-cell component in mitogenic assay of B-cell proliferation

	³ H-TdR incorporation (c.p.m.)
B cells	2,967
B cells + PWM	7,186
Irradiated T cells	1,314
B cells + T cells (irradiated; 0.2×10^6)*	20,302
+ IL-2 (containing both B- and T-cell stimulatory factors)†	
B cells + T cells (0.2×10^6)‡ + IL-2	22,066
B cells + T cells (0.02×10^6) + IL-2	19,353
B cells + T cells (0.01×10^6) + IL-2	21,309
B cells + T cells (0.005×10^6) + IL-2	21,314
B cells + T cells (0.002×10^6) + IL-2	20,223

B and T cells (where added) were incubated in 0.2 ml in microtitre wells for 48 h, then 1 µCi ³H-TdR was added to each well. After 24 h the cells were collected as described in Table 1 legend. The IL-2 preparations were added as 10% (v/v) of the final culture medium.

* These T cells were irradiated (2,000 rad) before co-cultivation.

† The IL-2 preparation was fractionated through DEAE-Sephadex as described in Table 1. This IL-2 (and that in Tables 3 and 4) contains both T- and B-cell stimulatory factors. The IL-2 preparation was shown to stimulate ³H-TdR incorporation of the magnitude demonstrated above in isolated B-cell preparations from multiple donors including the results shown in Table 1.

‡ These T cells and those in the following groups were not irradiated before co-cultivation.

was augmented in the presence of irradiated (2,000 rad) T cells and macrophage MØ. Testing of the interleukins revealed that IL-1 had no activity when added by itself to B cells. However, the IL-2 preparations, fractionated by either DEAE-Sephacrose chromatography or DEAE-Sephacrose plus Ultrogel chromatography, contained moieties that stimulated B-cell populations; these preparations showed a stimulation index equal to or greater than that seen in the same cells after PWM stimulation. To ensure that any potential residual T-cell contamination of the purified B-cell population was not responsible for the DNA synthesis seen in Table 1, increasing numbers of nylon wool-purified T cells were added to the purified B cells and then stimulated with the soluble factors contained in the IL-2 preparations fractionated through DEAE-Sephacrose chromatography (Table 2). As quiescent, non-lectin-activated T cells should not be stimulated by the lectin-free IL-2 preparations alone^{15,16}, we expected that the T cells would not account for the increased thymidine incorporation observed. The results (Table 2) show that addition of irradiated or non-irradiated T cells did not increase the level of ³H-TdR incorporation by B cells in response to the soluble factors in the IL-2 preparations.

As IL-2 has been shown to support T-cell blast proliferation *in vitro*, we next evaluated whether the IL-2 preparations would have any activity on PWM-stimulated B-cell blasts. We prepared B-cell blasts by adding PWM and irradiated syngeneic T cells to the B-cell population and incubating the cells in 5% CO₂ for 72–96 h. The irradiated T cells were then rosetted with sheep red blood cells (SRBC) and removed on Ficoll-Hypaque gradients while the MØ component was removed by adherence. The B-cell blast population was then stimulated with the IL-2 preparations containing B-cell growth factor for 72 h and ³H-TdR incorporation was measured during the last 24 h. Table 3 shows that soluble factors within DEAE-Sephacrose-fractionated IL-2 preparations stimulated significant ³H-TdR incorporation. Serial studies in which the blasts were re-stimulated with the B-cell growth factors at 72-h intervals, revealed that thymidine incorporation could be maintained for multiple reseeds and could be accounted for by B-cell stimulation alone. If the growth factor was deleted during these sequential studies, proliferation ceased. Furthermore, the percentage of

Table 4 Effect of soluble factors in Ultrogel-fractionated interleukin-2 preparations on B cells

Dilution of IL-2	³ H-TdR incorporation (c.p.m.)*	
	Quiescent B cells	B-cell blasts†
—	705 ± 86	2,438 ± 421
0.1%	606 ± 53	3,116 ± 1,001
0.5%	3,047 ± 943	16,067 ± 854
1.0%	3,340 ± 418	7,455 ± 2,983
5.0%	16,670 ± 4,868	18,465 ± 1,402
10.0%	9,639 ± 3,326	18,602 ± 862

The IL-2 preparation was purified by DEAE-Sephacrose and Ultrogel chromatography. Active material eluting from the DEAE-Sephacrose column was stabilized with polyethylene glycol¹³. The active fraction was then placed on an Ultrogel AcA54 column and the peak of T-cell activity eluting in the ~24,000-*M_r* range collected. This material (0.1% final volume) acting on lectin-stimulated T-cell blasts in a microtitre assay, gave 1,393 c.p.m. ³H-TdR incorporation at 72 h, whereas a 10.0% final volume gave 18,323 c.p.m. incorporated.

* Cells were placed in microtitre wells as described in Table 1 legend except that, 24 h before collection, they were labelled with 0.5 µCi ³H-TdR.

† B-cell blasts were prepared by stimulating purified B cells for 24 h in the presence of PWM. At the time of the microtitre assay, the PWM was washed from the cells and the IL-2 preparation added as above.

E-rosettable cells did not increase with time (Table 3), which strongly argues against the possibility that either T cells or null cells developing T markers account for the proliferation observed.

The results in Table 4, together with the data of Table 1, experiment 2, further demonstrate the presence of B-cell stimulatory factors in those IL-2 preparations that have been fractionated further by Ultrogel chromatography¹³. This material has been shown to contain a minimum of four bands discernible by gel electrophoresis. The factor(s) present in this fraction were again able to support the incorporation of ³H-TdR by purified B cells as well as purified T cells.

These data reinforce the concept that normal human B lymphocytes are auxotrophic, requiring accessory cell signals for activation^{17,18}. Most human B-cell mitogens such as PWM and *Staphylococcus* Protein A have been shown to be predominantly T-cell dependent in man^{19,20}. Various T-cell helper factors such as AEF²¹, LMF²², and T-cell replacing factor²³, all require T cells for their production. Reinherz *et al.*²⁴ have shown that LMF is a product of a monoclonal antibody-defined T helper subset. Macrophages are also apparently involved in B-cell activation by facilitating the interaction with helper T cells²⁵, and later by stimulating differentiation²⁶. Our study suggests that the interleukins as presently purified belong to this family of T-cell helper factors and factors within the preparations will stimulate ³H-TdR incorporation in both quiescent B cells as well as activated B-cell blasts in FCS.

The major question now is whether this proliferative response of B cells to the soluble factors in the interleukin 2 preparations is due to a nonspecific mitogenic effect of the putative T-cell-specific lymphokine or due to a co-purifying B-cell-specific growth factor. Experiments are being done to resolve this question by means of differential factor absorption from the IL-2 preparations. Initial observations indicate that when the IL-2 preparations are absorbed by a population of B cells and then mitogenically tested on lectin-activated T cells, the activity of the absorbed material is frequently more active than a preparation that has not been absorbed. The converse experiment, where the IL-2 preparations are absorbed by T cells and then tested on B cells, also reveals a frequent augmentation of the B-cell response compared with material that has not been absorbed (data not shown). Furthermore, absorption of the IL-2 preparations by B cells fails to remove T-cell-specific activity and T-cell absorption fails to remove the activity that acts on B cells. These preliminary studies suggest that either absorption leads to factor release in the media, or that the interleukin

Table 3 Effect of soluble factors in interleukin preparations on PWM-stimulated human B-cell blasts

Days in culture	Cells labelled	³ H-TdR incorporation (c.p.m.)	% Contamination by T cells
7	B cells	1,586	0
	B cells + IL-2	7,824	1
	(containing both T- and B-cell stimulatory factors)*		
11	B cells	1,693	0
	B cells + IL-2	51,672	2
14	B cells	1,336	0
	B cells + IL-2	28,256	0

* Purified B cells were mixed with an equal number of purified, irradiated (1,500 rad) T cells and 10% MØ by concentration. The cells were stimulated for 96 h with PWM (10 µl ml⁻¹) at which time the cells were depleted of adherent cells and E-rosettable cells as described in the text. The repurified B-cell blasts were then cultured in tubes (Falcon 3033) either in medium + FCS, or medium + FCS + the IL-2 preparation (1:10). The blasts were seeded at 5 × 10⁵ cells ml⁻¹ and re-fed with either medium-FCS alone or medium-FCS and the IL-2 preparations (containing both B- and T-cell stimulatory factors) every 72 h in association with re-feeding at the original concentration. In all cases, the B blasts were re-fed every 72 h; 24 h before re-feeding, an aliquot was labelled with 1 µCi ³H-TdR and the acid-precipitable material collected on Whatman GF/C filters and counted. Results represent the mean c.p.m. per assay point for a single experiment, with replicate experiments indicating the same trend; s.e.m. of cell population, <15%.

preparations contain co-purifying but separate B- and T-cell-specific stimulatory factors.

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Tumour promoters induce mitotic aneuploidy in yeast

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Tumour promoters, which complete the process of carcinogenesis initiated by subthreshold doses of carcinogens, can effect changes in gene regulation and cellular differentiation^{1,2}. Whether tumour promoters also induce genotypic changes is unclear. 12-O-tetradecanoylphorbol-13-acetate (TPA), a potent tumour promoter, is inactive in gene mutation assays^{3–5}, but has been reported by some^{6,7}, though not others^{8–10}, to induce sister chromatid exchanges, which may be cytological indications of recombinational events¹¹, in mammalian cells. However, TPA alone is not recombinogenic in yeast¹². We now report that in a genetic system using the yeast *Saccharomyces cerevisiae*^{13–15}, TPA fails to induce recombination but induces mitotic aneuploidy—a change in chromosome number which may lead to hemizyosity of recessive mutations or to phenotypic changes due to an alteration in gene balance. The ability of other agents to induce aneuploidy in this system also correlates with their tumour-promoting activity.

The compounds studied were assayed for their abilities to induce mitotic aneuploidy (see Fig. 1 for method) and crossing-over in the D₆ strain of *S. cerevisiae*^{13–15}, a diploid strain that carries a series of recessive, coupled markers on chromosome VII. The compounds tested included several promoters and

structurally related nonpromoters. All promoters tested for the ability to induce aneuploidy were positive, whereas non-promoters were negative (Table 1). Figure 2 plots the dose-response curve for TPA induction of aneuploidy, showing that dose-related increases in mitotic aneuploidy were obtained over the concentration range 0.02–5 µg ml⁻¹. Phorbol-12-13-didecanoate (PDD), another potent promoter in the phorbol ester series¹⁶, was also positive (Table 1). In contrast, no significant increases in aneuploidy were observed with phorbol, phorbol monoesters, 4-O-methyl-TPA or 4α-PDD, which are also inactive as promoters on mouse skin¹⁶.

Of particular interest is mezerein—a weak promoter, but as potent as TPA in inducing many biochemical and morphological effects in cells^{15,17}. In a two-stage promotion protocol on mouse skin¹⁷, mezerein is an incomplete promoter, that is, it is not effective in the first stage of promotion but is as effective as TPA in the second stage. In the concentration range 0.01–100 µg ml⁻¹, mezerein did not induce mitotic aneuploidy in our assay.

Other structurally unrelated promoters, including anthralin¹⁸, iodoacetic acid¹⁹, saccharin²⁰, oleic acid^{21,22} and lauric acid²², did induce mitotic aneuploidy. Stearic acid, a nonpromoter²², was inactive. Diethylstilboestrol, which may be a tumour promoter as well as initiator²³, was positive.

In contrast with the ability of the tumour promoters to induce mitotic aneuploidy, they had no effect on the frequencies of mitotic crossing-over. However, Fig. 3 shows that, in identical conditions, the incomplete promoter mezerein did induce mitotic crossing-over at concentrations >10 µg ml⁻¹ whereas TPA was ineffective. Mezerein was the only compound tested which showed significant activity in the crossing-over assays.

The expression of recessive mutations resulting from homozygosity produced by mitotic recombination has been suggested as an important step in tumour promotion⁶. However, with the exception of the incomplete promoter mezerein, none of the compounds tested was capable of inducing detectable levels of mitotic crossing-over in the yeast strain used here. In view of the relative ease with which mitotic crossing-over may be detected in yeast after treatment with complete carcinogens and initiating agents²⁴, it seems unlikely that the tumour promoters tested are capable of producing significant levels of mitotic recombination. The potential significance of the ability of mezerein to induce mitotic crossing-over is not clear.

Boutwell²⁵ has proposed that the promotion phase of carcinogenesis consists of at least two parts: phase I involving 'conversion' of the initiated cell and phase II the 'propagation' of the converted, initiated cell²⁵. Complete promoters, such as TPA, can induce both phases but some substances, such as mezerein, are incomplete promoters and can induce only the second phase¹⁷. Recently, Slaga and co-workers¹⁷ have confirmed this multi-stage model of tumour promotion and demonstrated that the conversion phase, like the initiation phase of carcinogenesis, requires only a single exposure to the cocarcinogen, although a dose-related increase occurs with additional exposures¹⁷. Furthermore, the conversion phase is irreversible, for at least 4 weeks after a single exposure to TPA (T. Slaga, personal communication). It is possible that the conversion phase of promotion requires a genetic change induced by the tumour promoter. The correlation of aneuploidy induction with promoters, nonpromoters and the incomplete promoter mezerein is consistent with a role for chromosome changes in this phase. The epigenetic changes induced by promoters in gene regulation and cellular differentiation are probably also important in overall tumour promotion.

We have shown an apparent correlation between the ability of a compound to induce mitotic aneuploidy in *S. cerevisiae* and its ability to act as a tumour promoter. While such a correlation may be coincidental, we suggest that it reflects a mechanistic relationship between chromosome aneuploidy and tumour promotion. Most chemical carcinogens have been shown to be capable of inducing point mutations in experimental systems²⁶; however no such activity has been demonstrated for tumour

Table 1 Correlation of tumour-promoting activity and induction of aneuploidy or crossing-over in assays using yeast strain D₆

Compound	Tumour promoting	Response in aneuploidy (conc. range in $\mu\text{g ml}^{-1}$)	Monosomic colonies per 10^6 viable cells Control Treated (in $\mu\text{g ml}^{-1}$)	No. observed colonies/no. plates	Response in mitotic crossing-over assay (conc. in $\mu\text{g ml}^{-1}$)	Red, cycloheximide-resistant, requiring adenine & methionine per 10^4 viable cells Control Treated (in $\mu\text{g ml}^{-1}$)	No. observed colonies/no. plates
Phorbol analogues							
1. TPA	Positive	Positive (0.02-5)	0- 3.7 $\pm$ 0.4 0.5- 41.5 $\pm$ 6.2	32/4 356/4	Negative (up to 100)	0- 4.7 $\pm$ 0.8 20- 4.5 $\pm$ 1.2	41/4 40/4
2. Phorbol-12,13-didecanoate (PDD)	Positive	Positive (1-10)	0- 4.2 $\pm$ 0.7 3- 52.2 $\pm$ 9.0	41/4 353/4	Negative (up to 50)	0- 7.0 $\pm$ 1.0 10- 8.8 $\pm$ 1.5	69/4 71/4
3. Phorbol	Negative	Negative (up to 50)	0- 4.2 $\pm$ 0.7 10- 5.2 $\pm$ 1.2	41/4 52/4	Negative (up to 50)	0- 7.0 $\pm$ 1.0 20- 8.3 $\pm$ 1.8	69/4 67/4
4. 12-O-tetradecanoyl-phorbol	Negative	Negative (up to 50)	0- 4.7 $\pm$ 1.4 20- 4.9 $\pm$ 1.2	44/4 45/4	Negative (up to 50)	0- 9.3 $\pm$ 1.3 30- 7.3 $\pm$ 1.1	87/4 58/4
5. Phorbol-13-acetate	Negative	Negative (up to 50)	0- 4.4 $\pm$ 1.6 20- 9.1 $\pm$ 2.2	37/4 79/4	Negative (up to 50)	0- 17.6 $\pm$ 2.5 10- 24.2 $\pm$ 2.9	147/4 172/4
6. 4-O-methyl-TPA	Negative	Negative (up to 50)	0- 4.4 $\pm$ 1.6 30- 5.1 $\pm$ 1.9	37/4 22/4	Negative (up to 50)	0- 17.6 $\pm$ 2.5 30- 19.1 $\pm$ 2.8	147/4 81/4
7. 4 α -PDD	Negative	Negative (up to 50)	0- 4.2 $\pm$ 0.7 20- 6.1 $\pm$ 0.8	41/4 52/4	Negative (up to 50)	0- 7.0 $\pm$ 1.0 40- 8.0 $\pm$ 1.1	69/4 51/4
Structurally unrelated compounds							
8. Anthralin	Positive	Positive (0.5-5)	0- 4.4 $\pm$ 1.6 1.5- 28.7 $\pm$ 4.4	37/4 162/4	Negative (up to 50)	0- 17.6 $\pm$ 2.5 40- 24.5 $\pm$ 5.6	147/4 54/4
9. Iodoacetic acid	Positive	Positive (5-50)	0- 4.2 $\pm$ 0.7 20- 21.8 $\pm$ 3.3	41/4 121/4	Negative (up to 100)	0- 7.0 $\pm$ 1.0 40- 11.7 $\pm$ 1.2	69/4 40/4
10. Oleic acid	Positive	Positive (100-500)	0- 4.2 $\pm$ 0.7 300- 16.3 $\pm$ 2.1	41/4 116/4	Negative (up to 50)	0- 7.0 $\pm$ 1.0 50- 11.1 $\pm$ 1.5	69/4 92/4
11. Lauric acid	Positive	Positive (10-200)	0- 9.1 $\pm$ 1.2 200- 29.7 $\pm$ 3.2	89/4 191/4	Negative (up to 50)	0- 12.7 $\pm$ 1.3 50- 13.1 $\pm$ 1.8	111/4 79/4
12. Stearic acid	Negative	Negative (up to 500)	0- 9.1 $\pm$ 1.2 400- 15.5 $\pm$ 2.4	89/4 130/4	Negative (up to 500)	0- 12.7 $\pm$ 1.3 300- 15.8 $\pm$ 3.4	111/4 47/4
13. Diethylstilboestrol	?	Positive (20-200)	0- 8.1 $\pm$ 2.0 50- 59.8 $\pm$ 10.2	91/4 134/4	Negative (up to 200)	0- 4.5 $\pm$ 0.6 25- 7.4 $\pm$ 1.9	51/4 27/4
14. Saccharin	Positive	Positive (200-400)	0- 10.6 $\pm$ 1.2 350- 153 $\pm$ 23.3	110/4 410/4	Negative (up to 500)	0- 7.5 $\pm$ 1.3 200- 9.4 $\pm$ 2.0	78/4 47/4
15. Mezerein	Incomplete	Negative (up to 100)	0- 3.7 $\pm$ 0.4 3- 4.7 $\pm$ 0.8	32/4 36/4	Positive (20-100)	0- 4.7 $\pm$ 0.8 30- 62.2 $\pm$ 9.2	41/4 122/4

Concentrations are those tested and, in most cases, represent those over which cell viability of tested cells was $> 10\%$ of the control value. At high levels of cell lethality, the reliability of the assay was reduced. A positive response was a threefold or greater increase in the frequency of aneuploid colonies in at least two separate experiments. In the case of phorbol-13-acetate, a small (twofold) increase was observed but the results obtained were not significantly different from the control values. A negative response was indicated if no significant increase in the frequency of aneuploid colonies or mitotic crossing-over occurred in either the left or right arm of chromosome VII. In the case of compounds 1, 2, 3, 4, 5, 6, 7, 9 and 13, no increases have been observed in the frequencies of induced mitotic recombination (including both gene conversion and crossing-over) in a variety of strains of yeast. The table also illustrates the maximum frequency of both induced aneuploidy and crossing-over observed in a single experiment. All the experiments used yeast cultures alone without the presence of an extracellular activating system involving rat liver microsomes (S9 mix). In the case of 12-O-tetradecanoyl-13-acetate, S9 mix reduced the induced frequency of aneuploid cells. The level of aneuploid cells in the control cultures was $3-9 \times 10^{-6}$ viable cells. Compounds were from: 1-6, Dr Peter Borchert (Chemical Carcinogenesis, Minnesota); 7, Consolidated Midland (New York); 9-13, Sigma; compound 8 was a gift from Dr Tom Slaga (Oak Ridge National Laboratory).

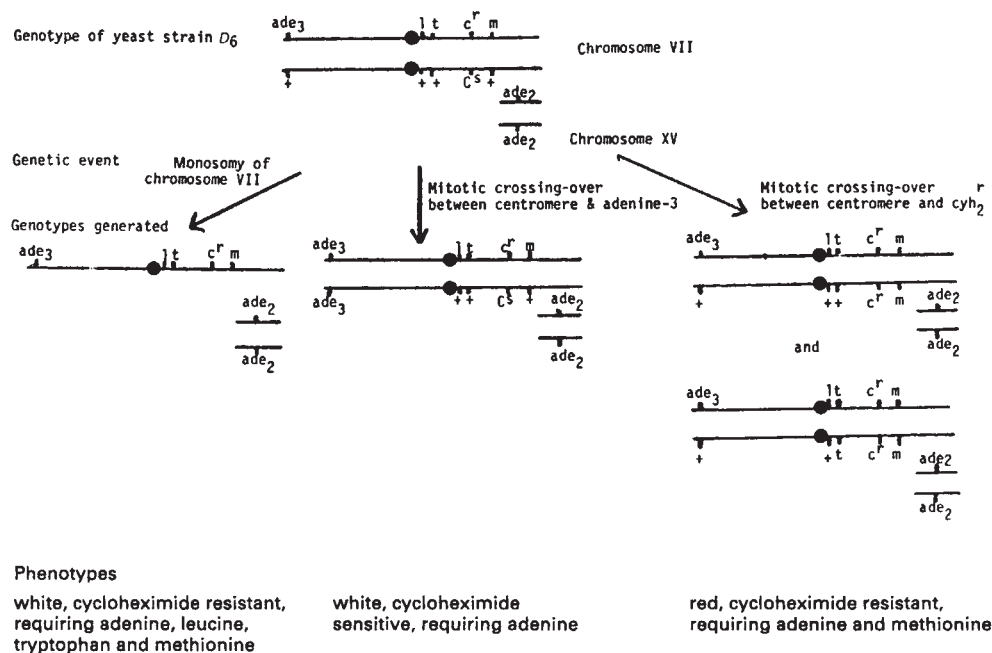


Fig. 1 Principles involved in the detection of mitotic aneuploidy in strain D₆. Cells homozygous for the ade_2 mutation require adenine for growth and produce red colonies; homozygosity of the ade_3 mutation, which occurs at an earlier step in adenine synthesis, leads to the production of white, adenine-requiring colonies. Homozygosity of all the markers on chromosome VII may also result from simultaneous crossing-over in both the left and right arm of chromosome VII; however, such events are measurable by the frequency of crossing-over assayed in both arms separately. The possible role of such multiple cross-over types in the origin of white, cycloheximide-resistant colonies has been extensively discussed elsewhere¹³. l , Leucine; t , tryptophan; c^r , cycloheximide resistance-2; m , methionine 13, C^r = cycloheximide sensitivity-2.

promoters, which seem incapable of direct interaction with DNA. Our data support the hypothesis that tumour promoters act by stimulating the expression of recessive mutations (presumably in regulatory genes) produced by an initiating agent or by altering gene balance, both of which could result from changes in chromosome number as a result of chromosome aneuploidy. Such changes in chromosome number have frequently been observed in the karyotypes of tumour cells²⁷ and the data reported here suggest they are causal in tumour

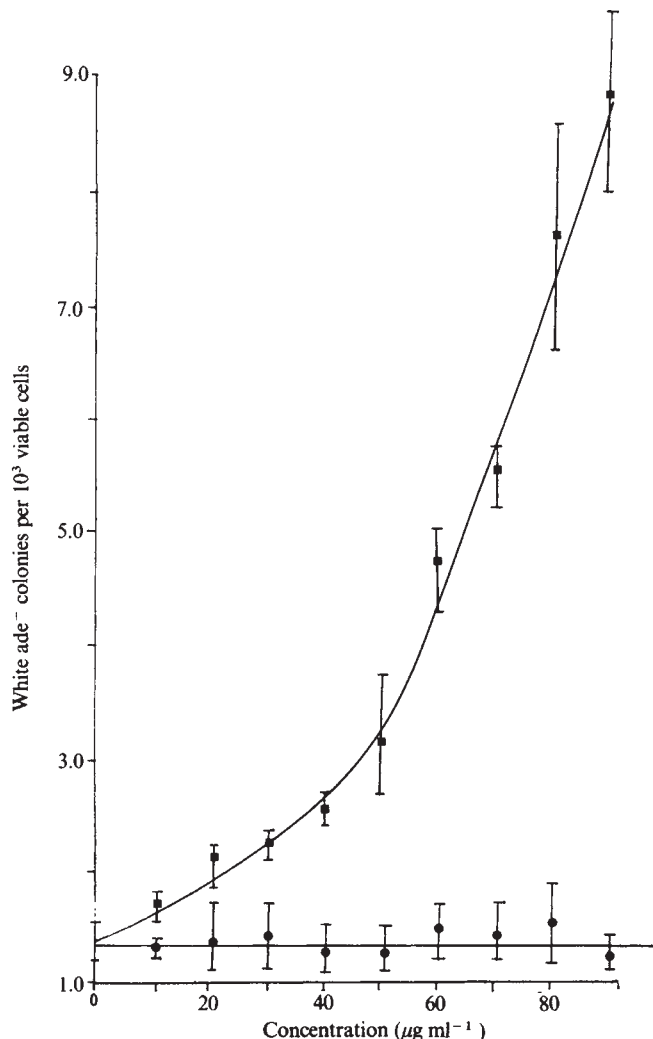


Fig. 2 Induction of mitotic aneuploidy in cultures of yeast strain D₆ after exposure to TPA (●) or mezerein (■). Cultures of strain D₆ were collected during exponential growth, washed and resuspended in pH 7.0 phosphate buffer at a cell concentration of 10^7 ml^{-1} . Cell samples (2 ml) was incubated at 30 °C in sealed bottles in a shaking water bath for 6 h in the presence of TPA or mezerein at concentrations of up to $5 \mu\text{g ml}^{-1}$ (both compounds were prepared as stock solutions in dimethyl sulphoxide). Complete growth medium (2 ml) was then added to each bottle, and shaking and incubation continued for a further 18 h. In the experiment shown, 18 ml of growth medium were added. Cultures were washed three times by centrifugation, resuspended in sterile saline and plated after suitable dilution on yeast complete medium to score cell viability and on yeast complete medium plus $2 \mu\text{g ml}^{-1}$ of cycloheximide to score white, cycloheximide-resistant, monosomic ($2n-1$) colonies. All treated cultures were examined microscopically after washing and in no case was there evidence of sporulation amongst the diploid cells. Viability plates were scored after 5 days and the frequency of monosomic colonies after 10 days of incubation at 28 °C. Representative samples of white, cycloheximide-resistant colonies were replicated onto selective minimal plates to determine the expression of the recessive markers on chromosome VII of strain D₆. All compounds reported here as inducing mitotic aneuploidy gave rise to white, cycloheximide-resistant colonies requiring adenine, leucine, tryptophan and methionine, as would be expected of cells which have lost one copy of chromosome VII. Details of the genetic basis of this assay are described in ref. 14. It is technically possible that some homozygous colonies arose from meiotic recombination during the treatment period; however, in none of the cultures was meiosis observed and increased recombination would have been observable in our crossing-over assay.

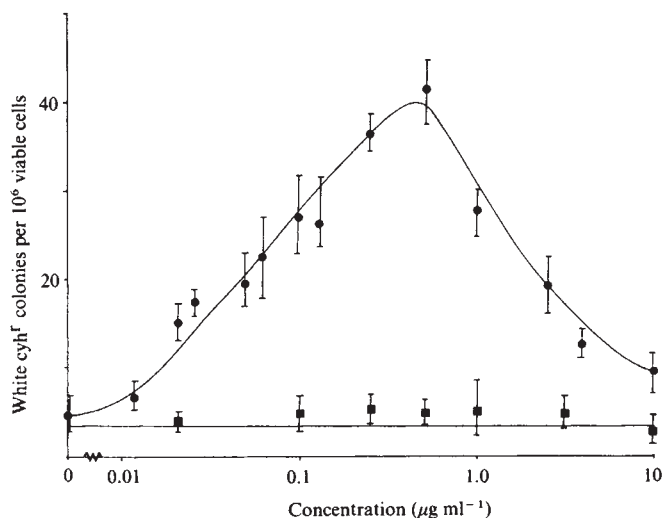


Fig. 3 Induction of mitotic crossing-over in cultures of yeast strain D₆ after exposure to TPA (●) and mezerein (■). Cultures were exposed to either compound at concentrations of up to $100 \mu\text{g ml}^{-1}$ in the conditions described in Fig. 2 legend. After treatment, cultures were plated for cell viability, for the induction of white, cycloheximide-resistant, monosomic colonies and for the frequency of white, cycloheximide-sensitive colonies produced on yeast complete growth medium at dilutions giving 10 times more viable cells than on the viability plates. All treated cultures were examined microscopically after washing and in no case was there any evidence of sporulation among the diploid cells. White, cycloheximide-sensitive colonies were replicated onto supplemented (with leucine, tryptophan and methionine) minimal plates. More than 90% of the white colonies are adenine requiring and result from mitotic crossing-over between the centromere and the *adenine-3* gene on the left arm of chromosome VII. Assays of mitotic crossing-over were also performed using the *cycloheximide-2* gene on the right arm of chromosome VII with similar results.

development. The changes in chromosome aneuploidy would readily fit the multi-stage models of carcinogenesis.

The data presented here do not indicate whether the assay of induced chromosome aneuploidy in yeast will be useful in detecting tumour promoting chemicals. Such a conclusion awaits the demonstration that chromosome aneuploidy is also induced in mammalian cells by tumour promoters.

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Isolation of I-A subregion-like molecules from subhuman primates and man

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Immune response-associated (Ia) molecules play a major part in cellular interactions that initiate an immune response. Two families of Ia molecules, determined by the I-A and I-E subregions of the murine major histocompatibility complex (MHC), have been isolated and characterized in mice¹. I-A and I-E molecules each consist of two polypeptide chains (molecular weights (M_r s) of 26,000 and 35,000 for I-A and 24,000 and 32,000 for I-E) that are noncovalently associated². Their large subunits are designated A_α and E_α , respectively, and their small subunits A_β and E_β . Despite the similarity in their subunit composition, the I-A and I-E molecules are structurally distinct according to peptide mapping and partial N-terminal sequence analysis^{3,4}. Although the structural homologue of murine I-E molecules has been identified in man and is known as HLA-DR⁵, the I-A equivalent has never been described. In an attempt to identify the human analogue of the murine I-A molecule, murine monoclonal antibodies were produced after immunization with a mixture of three putative human cell lines. We report here that one of these antibodies reacts with I-A-like molecules from two cell lines, GM3158, which after karyotype analysis was shown to have originated not from man but from a marmoset, and GM3163, a cell line of human origin.

Monoclonal antibodies to cell surface molecules were produced by immunizing BALB/c mice with a mixture of the cell lines GM3107, GM3158 and GM3163 (obtained from the Human Mutant Cell Repository, Camden, New Jersey) and fusing the immune splenocytes with SP2/O-Ag cells. After appropriate screening by indirect immunoprecipitation, several positive clones were identified—two of these, designated SG157 and SG171, were analysed further. When tested with a lentil lectin-purified glycoprotein fraction from cell line GM3158, the monoclonal antibodies precipitated molecules having two, slightly different profiles after SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 1). SG157 precipitated products with SDS-PAGE profiles characteristic of human HLA-DR antigens: two polypeptides of M_r s ~29,000 and 34,000, respectively with the smaller subunit (β) displaying the characteristic retardation in migration rate in reducing conditions². In contrast, SG171 precipitated two molecules of different molecular weights (~30,000 and 33,000); in reducing conditions these merge into a single peak with an apparent M_r of 33,000. Peptide map comparisons (Fig. 2) of the molecules isolated with SG157 and SG171 reveal striking differences which suggest that, despite similarities in their molecular weights, the molecules detected by SG157 and SG171 are unrelated.

The molecules binding to SG157 and SG171 were identified by limited amino acid sequence analysis (Table 1). The two polypeptide chains reacting with SG157 show a high degree of sequence identity, 9 of 11 residues (82%), with the two polypeptide chains of the murine I-E molecule and thus represent the marmoset analogue. In contrast, the two polypeptide chains reacting with SG171 are homologous to the murine I-A molecule. The large (α) subunit of the molecule binding to SG171 is identical to a prototype of the large subunit of murine I-A molecules (A_α) in 5 of 8 sequence residues that can be compared, provided a gap is inserted at the amino terminus.

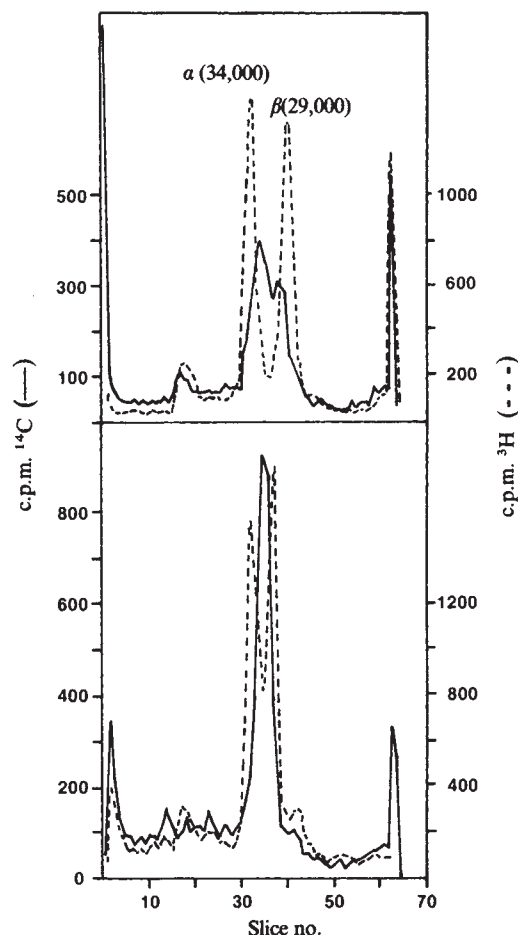


Fig. 1 SDS-PAGE profiles of molecules isolated from cell line GM3158 with SG157 (---) and SG171 (—) monoclonal antibodies in non-reducing (top) and reducing (bottom) conditions. Cells (2×10^8) were labelled in culture with either ^3H - or ^{14}C -phenylalanine and their glycoprotein components were isolated as described elsewhere⁸. The relevant molecules were isolated by adsorption of the radiolabelled glycoprotein fractions on to an immunoabsorbent column consisting of monoclonal antibody (SG157 or SG171) covalently coupled to Sepharose-4B, followed by elution with 0.05 M diethylamine. The eluted material was lyophilized, dissolved in water and acetone precipitated. The precipitates were dissolved in Laemmli¹⁴ non-reducing sample buffer and ^{14}C - and ^3H -labelled material was mixed in a 2:1 ratio. One half of the sample was reduced with 2-mercaptoethanol. The samples were electrophoresed on 12.5% polyacrylamide gels and the gels were sliced into 1 mm thick sections. After overnight incubation in water the entire slice, as well as the eluted material, was counted in a liquid scintillation counter. Radioactivity (c.p.m.) was corrected for ^3H and ^{14}C channel spillover.

Furthermore, the small subunit (β) is identical to that of the murine I-A molecule (A_β) in 6 of 7 sequence residues that can be compared. The combined degree of homology, 11 of 15 residues (73%), is comparable with that observed between other MHC products of different species (that is, mouse and man)^{5,6}, and strongly suggests that the molecule reacting with SG171 is the homologue of the murine I-A molecule.

The marmoset I-A molecule differs in several respects from its murine counterpart. Although both consist of two noncovalently associated subunits of similar molecular weight, the two subunits of the murine I-A molecule are generally better resolved on SDS-polyacrylamide gels². In addition, whereas reducing conditions do not appreciably alter the migration rate of either subunit of murine I-A molecules compared with those of the unreduced forms when analysed by SDS-PAGE, the small

Table 1 Partial NH₂-terminal sequences of I-A- and I-E(DR)-like molecules isolated from cell line GM3158

	Residue																		Homology
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
(SG157) α	Ile							Ile				Phe	Tyr	Leu	Val				6/7
Murine I-E α	Ile	Lys			His	Thr	Ile	Ile		Ala		Phe	Tyr	Leu	Leu	Pro			
(SG157) β	[]							Phe				Leu						Phe	3/4
Murine I-E β	Val	Arg		Ser	Arg	Pro		Phe	Leu		Tyr	Ser	Lys	Ser				Phe	
												Val	Thr						
(SG171) α	[]			Ile	Val	Ala						Tyr		Val			Tyr		5/8
Murine I-A α				Ile	—	Ala			Val		Val	Tyr		—		Val	Tyr		
									Val		Phe								
(SG171) β							Phe	Val	—		Phe			Phe		Tyr	Phe		6/7
Murine I-A β		Ser			Arg	His	Phe	Val	Tyr		Phe	Pro	Pro			Tyr	Phe		
									Val			Ser	Lys						
									Phe										

Cells were radiolabelled *in vitro* with several ³H-amino acids and SG157 and SG171 molecules were isolated and purified as described in Fig. 1 legend. Proteins were sequenced and residues identified as described elsewhere⁸. Boxed residues represent identities between species and dashes indicate presence of residue found in one species but absent from the other. The murine sequence data were compiled from refs 3, 5, 12 and 13.

subunits of marmoset I-A- and I-E-like molecules migrate more rapidly in non-reducing conditions. Furthermore, one must insert a gap at the N-terminus of the large subunit of the marmoset I-A-like molecule to align it with the large subunit of the murine I-A molecule. A similar requirement applies to the I-E(DR)-like molecule, for which a gap must be inserted at the N-terminus of the small subunit to align it with the small subunit of the murine I-E molecule⁷. The significance, if any, of these similarities between I-A and I-E-like molecules in marmosets is unknown.

The recognition that GM3158 is a marmoset cell line explains our previous observation that the DR α chain isolated from GM3158 differs structurally from the DR α chains isolated from two human cell lines, GM3107 and GM3163 (ref. 8). Although the DR α subunit is essentially invariant in structure within a species (mouse or man)⁹⁻¹¹, its structure obviously varies between species.

Identification of an I-A-like molecule in subhuman primates strongly suggests that this molecule also exists in man. Indeed,

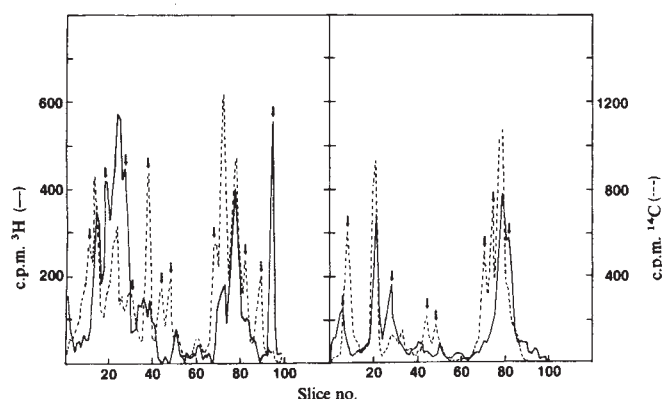


Fig. 2 Tryptic peptide comparisons of α (left) and β (right) polypeptides isolated from GM3158 with monoclonal antibodies SG157 (---) and SG171 (—). ³H- and ¹⁴C-phenylalanine-labelled molecules were isolated with SG171 and SG157, respectively, and the component α and β subunits separated by SDS-PAGE as described in Fig. 1 legend. Equivalent c.p.m. of ¹⁴C (α or β)- and ³H (α or β)-polypeptides were combined. After complete reduction, alkylation and trypsin-TPCK (tosyl-phenylethyl-chloromethyl-ketone) digestion, the peptides were separated on isoelectrofocusing gels as described elsewhere⁸. The gels were sliced into 1-mm sections, incubated in water overnight and measured for radioactivity, which was plotted against slice number after correcting for ³H and ¹⁴C channel spillover. Arrows denote peptide differences.

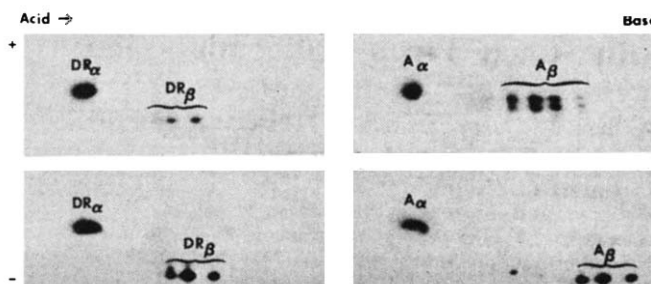


Fig. 3 Two-dimensional gel electrophoresis of DR- and I-A-like molecules isolated from cell lines GM3158 and GM3163. The DR- and I-A-like molecules were purified by immunoadsorption as described in Fig. 1 legend, except that Triton X-100 instead of deoxycholate was used in the elution step. The glycoprotein extract from GM3163 was first depleted of SG157-reactive material before being combined with the SG171 immunoadsorbent. The isolated material was analysed as described elsewhere¹⁵ except that non-reducing conditions were used throughout. The DR- and I-A-like molecules isolated from marmoset (top row) and man (bottom row) are distinguishable primarily by the difference in isoelectric points of the DR β and A β polypeptides. The marmoset and human I-A-like molecules have ~90% amino acid sequence homology (to be published).

preliminary results indicate that SG171 also reacts with an I-A-like molecule from some human cell lines. Two-dimensional gel analysis of molecules isolated from cell line GM3163 (confirmed by karyotype analysis to be of human origin) with SG157 and SG171 revealed DR- and I-A-like molecules respectively (Fig. 3). As in the marmoset, the A β polypeptide is more basic than the DR β polypeptide. However, in contrast to the marmoset, human DR- and I-A-like molecules have almost identical molecular weights. It is also intriguing to note that the A α and DR α subunits have not only identical molecular weights but also isoelectric points, suggesting that despite a lack of similarity in their NH₂-terminal amino acid sequences, they may be evolutionarily related.

The similarity in molecular weights of the DR- and I-A-like molecules in man may explain why others have failed to identify the latter. It is likely that I-A-like molecules have been isolated but mistakenly identified as DR because of their similar migration rates when analysed by SDS-PAGE.

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Isolation of a new type C retrovirus (HTLV) in primary uncultured cells of a patient with Sézary T-cell leukaemia

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Retroviruses have been isolated from many animal species and several have been shown to be the aetiological agents of naturally occurring leukaemias, lymphomas and sarcomas (for recent reviews see ref. 1). There is evidence that these viruses or components of them are present in humans, but the isolation and analysis of retroviruses from human cells and evidence for an association with disease have been difficult to demonstrate (see refs 2, 3). We now report the isolation and characterization of a new type C retrovirus (HTLV_{MB}) from a patient with a cutaneous T-cell leukaemia (Sézary syndrome). Analysis of the proteins and nucleic acids of this retrovirus isolate indicates that it is closely related to HTLV_{CR}, a retrovirus recently isolated⁴ and characterized^{5–7} from a patient with cutaneous T-cell lymphoma (mycosis fungoides) but distinct from known animal retroviruses. Proteins and nucleic acids of HTLV were also identified in the fresh (uncultured) Sézary leukaemic blood cells from which the second isolate was derived.

Retrovirus particles are usually not demonstrable until cells are successfully grown in culture. As T cells are often target cells for transformation by type C retroviruses, it is necessary to grow human T cells in continuous culture to determine whether they contain these viruses. The discovery of T-cell growth factor (TCGF)^{8,9} and its recent purification from human lymphocyte conditioned media¹⁰ have now made it possible routinely to grow normal T cells for long periods if the TCGF is added periodically to lectin- or antigen-activated lymphocytes. Neoplastic cells can be separated and grown in culture without normal T cells by using purified TCGF because many neoplastic T cells differ from normal T cells in not requiring *in vitro* activation by lectin to interact with TCGF¹¹. Some cultured neoplastic T cells become independent of exogenous TCGF^{11,12}, probably because they become constitutive TCGF producers¹³. The use of TCGF has thus enabled neoplastic cell lines to be established and their retrovirus-producing properties analysed.

Mycosis fungoides and Sézary syndrome are two distinct clinical variants of a group of neoplastic diseases referred to as

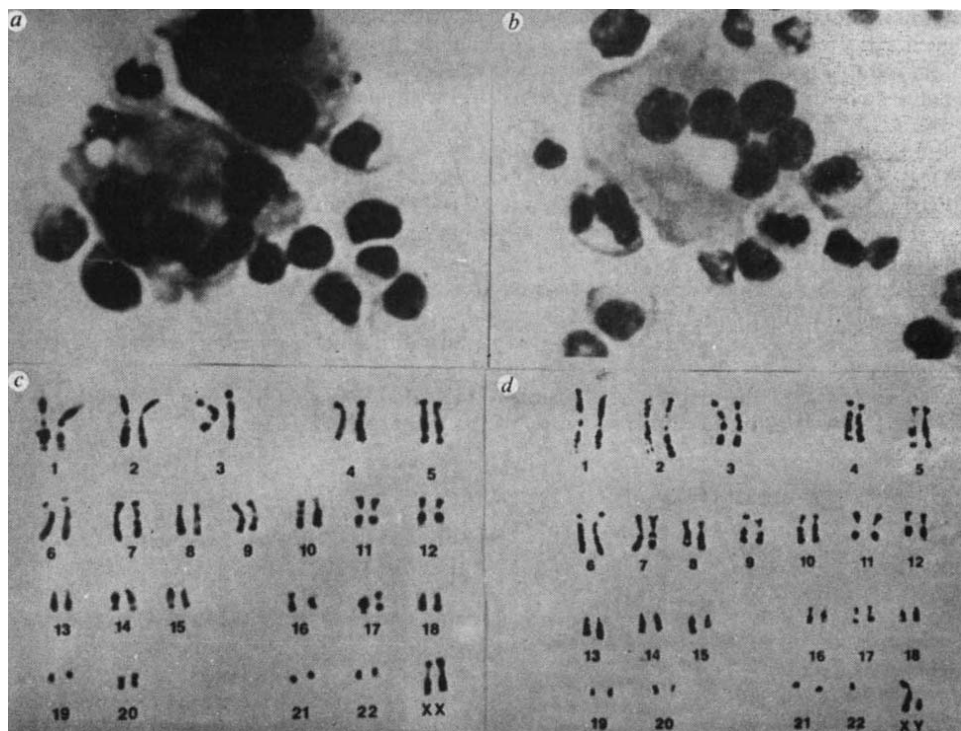
cutaneous T-cell lymphomas and leukaemias respectively¹⁴. The detailed clinical history of patient C.R. with mycosis fungoides has been published elsewhere⁴. The cell line, HUT102, was established from neoplastic T cells derived from his right inguinal lymph node¹², using crude TCGF⁸. One year later, the cell line CTCL-3 was established from his peripheral blood using a lectin-free partially purified TCGF¹¹. Patient M.B. was a 64-yr old black female with the leukaemic phase of Sézary syndrome. Examination of her peripheral blood revealed a white blood cell count of 300,000 mm⁻³ with a differential of 72% lymphocytes, 35% of which had markedly convoluted nuclei, characteristic of Sézary leukaemic cells. The cell line CTCL-2 was established¹¹, again using partially purified TCGF¹⁰, from a sample of her peripheral blood.

The characteristics of the three cell lines are similar and have been published in detail previously^{11,12}. HUT102 and CTCL-2 are now grown independently of exogenous TCGF, while CTCL-3 remains dependent on its addition. Their morphology is that of atypical mono- and multi-nucleated lymphoblasts (Fig. 1a,b). Typical of T lymphoblasts, they form rosettes with sheep erythrocytes and are negative for Epstein-Barr virus nuclear antigen. Their histochemical staining pattern (negative for myeloid stains but positive for α -naphthyl acetate esterase and acid phosphatase) and lack of terminal transferase activity are compatible with published reports on fresh cells from their disease of origin. Karyotypes performed on the cultured cells (Fig. 1c,d) are the same as those performed on the original fresh tissue source and have remained constant throughout culture (2 yr).

HUT102 and CTCL-3 are now constitutive producers of type C retrovirus particles (HTLV_{CR}), while fresh peripheral blood lymphocytes from patient C.R. produced particles only after induction with 5-iodo-2'-deoxyuridine (IUdR). Similarly, CTCL-2 cells have produced typical type C particles (Fig. 2) from the earliest passage studied (passage 2), but only after induction with IUdR. Figure 3 summarizes the isolations of these viruses. As the cell lines are relatively poor producers of virus, large amounts of cells must be cultured for analytical studies. Typically, 20–50 l of cells (10⁶ per ml) are grown in RPMI-1640 with 10% fetal calf serum, and in the case of CTCL-2, the cells are also treated with IUdR (20 μ g ml⁻¹) for 24 h. The virus-releasing cells are then centrifuged, resuspended in fresh media and the media collected 72 h later. Virus particles are concentrated and purified by sequential banding in sucrose, pelleting through 30% glycerol and finally isopycnic banding in a continuous sucrose gradient. The fractions containing HTLV_{MB} particles are identified by electron microscopy and by assaying for viral reverse transcriptase. These methods have been previously described for HTLV_{CR} (ref. 4). As with HTLV_{CR} and most other retroviruses, concentrated HTLV_{MB} particles band at a density of 1.16 g ml⁻¹ and are associated with an endogenous DNA polymerase activity. The polymerase shows a preference for the synthetic template primers poly(A)-oligo(dT) and poly(C)-oligo(dG) over poly(dA)-oligo(dT), characteristic of viral reverse transcriptase¹⁵. Like HTLV_{CR} reverse transcriptase⁶, HTLV_{MB} reverse transcriptase has a preference for Mg²⁺ with poly(A)-oligo(dT) and poly(C)-oligo(dG) (data not shown).

Analysis of isolated core structures from HTLV_{CR} has shown that a 24,000 molecular weight protein (p24) is a major component of the viral cores⁷; this protein has been purified⁷. In a competitive radioimmunoassay using labelled, purified HTLV_{CR} p24 and an antibody against whole disrupted HTLV_{CR}, all known animal retroviruses tested to date have failed to compete, whereas purified HTLV_{CR} and cells producing HTLV_{CR} do compete⁷. Both HTLV_{MB} and HTLV_{CR} isolates competed in the HTLV_{CR} p24 assay (Fig. 4a), suggesting a high degree of relatedness between p24 of the two isolates. Furthermore, cell lysates of cultured, IUdR-induced CTCL-2 T lymphoblasts also compete whereas lysates of normal phytohaemagglutinin (PHA)-stimulated human T lymphoblasts do not (Fig. 4a, b). These findings indicate that p24 is expressed in

Fig. 1 Morphology and karyotypes of CTCL-2 and HUT102 cells. Light microscopic appearance of cultured HUT102 (a) and (b) CTCL-2 cells. Cells were pelleted in a cyto-centrifuge and stained with Wright-Giemsa. Both cell lines are morphologically similar, with atypical mono- and multinucleated lymphoblasts. Karyotype analysis was performed as previously described¹¹. CTCL-2 metaphases (c) were those of a normal diploid human female (46, XX), while HUT102 metaphases (d) were predominantly those of a pseudodiploid human male (46, XY, minus chromosome 22, plus a minute).



CTCL-2 cells after IUdR induction but not in normal human T lymphoblasts with or without IUdR induction.

Liquid molecular hybridization experiments⁵ on HTLV_{CR} showed that: (1) HTLV_{CR} ³H-cDNA hybridized almost completely (90%) to its own 70S RNA with kinetics consistent with the genetic complexity of other retroviruses, (2) HTLV_{CR} was not significantly related to a wide variety of animal retroviruses (types B, C and D), including bovine leukaemia virus (BLV) and previously described endogenous and horizontally transmitted viruses from non-human primates, and (3) nucleic acid sequences related to ³H-cDNA or ¹²⁵I-70S RNA HTLV_{CR} were not found in DNA from several normal human tissues.

In liquid hybridization experiments to assess the nucleotide sequence relatedness of HTLV_{CR} and HTLV_{MB}, 90% of the ³H-cDNA from HTLV_{CR} hybridized to HTLV_{CR} 70S RNA and 54% to RNA from cultured HUT102 cells. In contrast, there was no significant hybridization (<10%) to RNA from phytohaemagglutinin (PHA)-stimulated human peripheral blood T lymphocytes from 20 pooled normal donors, to RNA from a normal human embryonic cells line, or to 70S RNA from several other type-C viruses (Table 1). As with cytoplasmic RNA of HTLV_{CR}-producing cells, ³H-cDNA prepared from HTLV_{CR} also hybridized to IUdR induced CTCL-2 cytoplasmic RNA (45%). The kinetics of hybridization indicate that CTCL-2 cytoplasmic RNA contains about 0.5% HTLV-specific sequences by weight (Fig. 5). The *T_m*s of the resultant hybrids are identical with both sets of cell RNA (Fig. 5 inset).

³H-cDNA from purified HTLV_{MB} hybridized 43% to HTLV_{CR} 70S RNA but only 19% to 70S RNA from avian myeloblastosis virus (AMV), providing further evidence that HTLV_{MB} is closely related to HTLV_{CR}. (The 19% value with AMV 70S RNA is indicative of a high background with this cDNA, not a specific relatedness to AMV.) As we had insufficient HTLV_{MB} to prepare 70S RNA, we cannot say whether 43% represents the maximum ability of this cDNA to hybridize to the 70S RNA of HTLV_{CR} or whether the HTLV_{MB} and HTLV_{CR} have some nonrelated sequences. The data clearly show, however, that the two isolates are at least closely related. This relationship is now being investigated further.

A frozen portion of the original (uncultured) neoplastic peripheral blood cells used to establish cell line CTCL-2 from patient M.B. was examined for the presence of HTLV_{CR}-related

proteins and nucleic acid sequences. Proteins from cell lysates of these fresh cells competed effectively in the radioimmunoassay for HTLV_{CR} p24, whereas extracts from PHA-stimulated normal human T lymphoblasts did not (Fig. 4b). Cell proteins were fractionated by phosphocellulose column chromatography as described elsewhere⁷ and the fractions eluting from a NaCl gradient at 150–300 mM were positive for HTLV p24 as assessed by competition with ¹²⁵I-p24 of HTLV in competition radioimmunoassays (Fig. 4b). These results indicate that a protein antigenically related or identical to HTLV_{CR} p24 was

Table 1 Nucleic acid relatedness of retrovirus isolates, HTLV_{CR} and HTLV_{MB}

Nucleic acid	% Hybridization with HTLV _{CR} ³ H-cDNA
RNA	
Viral	
HTLV _{CR} 70S	90
GALV _H 70S	13
BaEV 70S	7
AMV 70S	4
Cellular	
HUT102 (HTLV _{CR})	54
CTCL-2 (HTLV _{MB})	45
PHA-stimulated normal human lymphoblasts	9
Human embryonal carcinoma cells	6
DNA	
HUT102 (HTLV _{CR})	45
CTCL-2 (HTLV _{MB})	21
Fresh leukaemic cells of patient M.B.	17
PHA-stimulated normal human lymphoblasts	8
Normal human tissues	3–11*

HTLV ³H-cDNA (500–1,000 c.p.m.) was hybridized to 1 µg 70S viral RNA, 200–300 µg cellular cytoplasmic RNA or 600 µg cellular DNA as described previously^{4,20}, and the per cent hybridization determined by S₁ nuclease digestion. The values indicated are maximum plateau values in the conditions used. The heterologous viral 70S RNAs tested included those from gibbon ape leukaemia virus, Hall's Island strain (GALV_H)²², baboon endogenous virus (BaEV) and avian myeloblastosis virus (AMV). The cell lines HUT102 and CTCL-2 are the abnormal T-lymphoblast cell lines derived from patients C.R. and M.B., respectively. The viruses produced by these two cell lines are shown in parentheses. PHA-stimulated lymphoblasts are human peripheral blood leukocytes pooled from 20 normal donors and stimulated in short-term culture (72 h) with PHA prepared as previously described²³.

* Mean of 30 samples = 8%, s.d. = 4%.

present in the original uncultured tumour cells. The IuDR-induced cultured CTCL-2 cell lysates competed at a lower protein input (Fig. 4a) than the fresh lysate (50% competition for cultured cell lysate at 0.8 μ g protein compared with 150 μ g for the fresh cells), indicating a higher p24 concentration in cultured cells.

HTLV_{CR} ³H-cDNA hybridizes to DNA from both cultured HUT102 (45%) and CTCL-2 (21%) cells, but not to DNA from normal human tissues nor to DNA from a human embryonal carcinoma cell line (Table 1). In addition, HTLV_{CR} cDNA hybridized 17% to DNA from the original fresh peripheral blood neoplastic T cells of patient M.B. (Table 1), whereas hybridization of HTLV ³H-cDNA to DNA of normal human tissues was 3–11% (>30 samples tested), mean 8 ± 4 s.d. The differences between the degree of hybridization of HTLV_{CR} cDNA to DNA of cultured cells from patient C.R. and DNA of

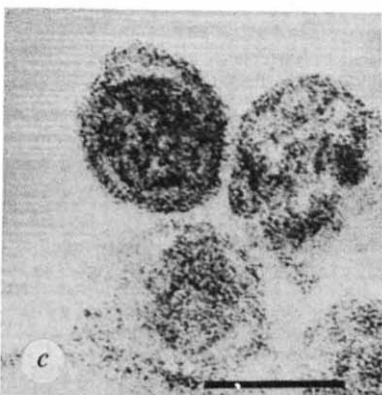
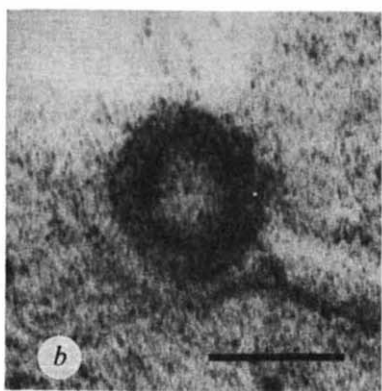
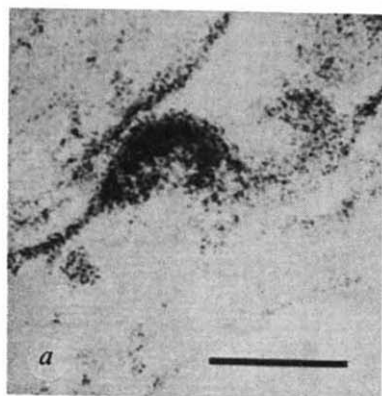


Fig. 2 Electron micrographs of HTLV_{MB}. Thin section electron microscopy was performed on CTCL-2 cell pellets as previously described⁴. Shown here are typical budding (a) and extracellular immature (b) and mature (c) type C particles found in clumps adjacent to the T lymphoblasts. Scale bar, 100 nM.

Strain from C.R.

Strain from M.B.

1. HUT102

P4

IuDR

P50

P56

Constitutive

2. Fresh lymphocytes

IDUR

3. CTCL-3

P2

Constitutive

1. CTCL-2

P2

IuDR

•

•

•

•

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Fig. 3 Schematic representation of HTLV isolation from the fresh and cultured cells from two patients (C.R. and M.B.) with cutaneous T-cell lymphoma and leukaemia, respectively. The dots indicate that the cell lines continue to produce virus in succeeding culture passages. The p numbers refer to different passages of the cells in culture. IuDR refers to requirement of the cells for treatment with IuDR for release of HTLV, while 'constitutive' indicates that the cells either subsequently or originally produced HTLV without IuDR (see text for further details).

fresh and cultured cells from patient M.B. again suggest that some non-related nucleic acid sequences exist in the HTLV isolates. The specificity of this hybridization is emphasized by the fact that ³H-cDNA from Simian Sarcoma virus, baboon endogenous virus, BLV, Rauscher murine leukaemia virus, squirrel monkey virus and langur endogenous virus failed to hybridize to CTCL-2 cytoplasmic RNA (data not shown). The hybridization of HTLV_{CR} cDNA to DNA from the fresh Sézary T cells was significantly less than with DNA from cell lines

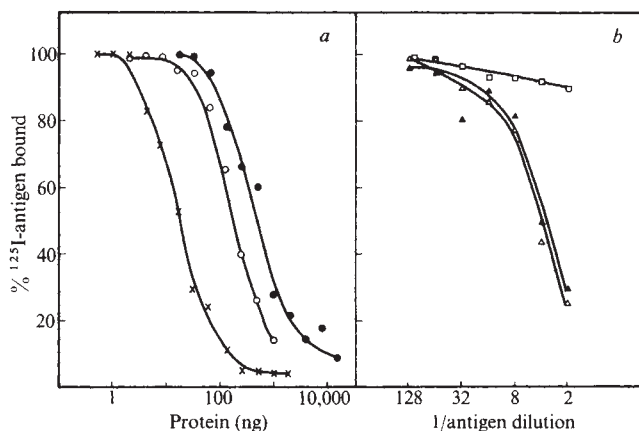


Fig. 4 Relatedness between p24 from HTLV_{CR} and from HTLV_{MB} and evidence for this p24 in fresh blood cells of patient M.B. HTLV p24 was purified by a combination of phosphocellulose chromatography and gel filtration in Biogel p60 as described earlier⁷. The proteins were iodinated by the chloramine-T method. The radioimmunoassays used 7,000 c.p.m. of ¹²⁵I-labelled HTLV p24 and a limiting dilution of a rabbit antibody to HTLV⁷. The viruses and cells used in the assays were solubilized in buffer containing 10 mM phosphate pH 7.5, 0.8 M NaCl, 0.5% Triton X-100 and 0.5 mM phenyl methyl sulphonyl fluoride. The competing unlabelled antigens used in the assay are: (in panel a) ×, HTLV_{CR}; ○, HTLV_{MB} from cultured CTCL-2 cells; ●, extracts from cultured CTCL-2 cells; and (in panel b) Δ, fresh blood cells of patient M.B.; ▲, pool of the proteins from fresh blood cells of patient M.B. partially purified by phosphocellulose chromatography; □, extracts from cultured normal human T cells.

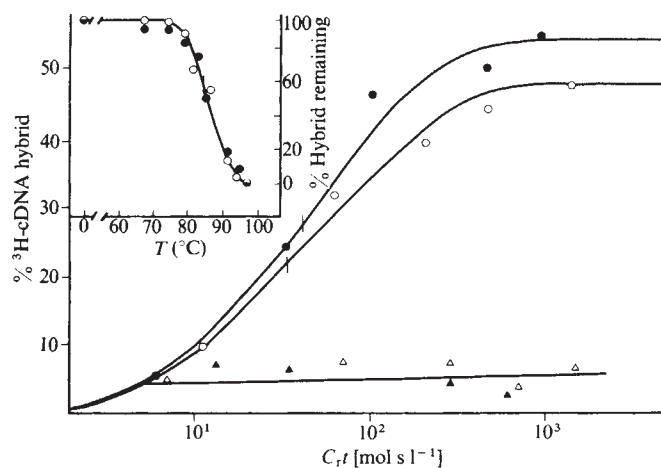


Fig. 5 Nucleic acid relatedness of HTLV_{MB} to HTLV_{CR}. HTLV_{CR} ³H-cDNA was prepared as previously described⁵, hybridized to 200 µg of the indicated cytoplasmic RNA in 50% formamide-3X SSC (1X SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7) with 0.05% SDS and 1 mM diethylpyrocarbonate, and the amount of hybrid assayed by S₁ nuclease digestion. The cDNA was hybridized to the indicated C, t. Values were normalized to the maximum value with HTLV 70S RNA (actual value 90%). Inset: cDNA was hybridized to a C, t of 500, diluted 20-fold with 2X SSC, and incubated at the indicated temperature for 5 min. Hybridization is normalized to 100% for each hybrid (actual values 48% and 44%, respectively). ●, HUT102 cytoplasmic RNA; ○, IUDR-treated CTCL-2 cytoplasmic RNA; △, human embryonic cell line cytoplasmic RNA; ▲, PHA-stimulated normal human blood T lymphoblasts obtained as previously described²³.

infected by and producing HTLV. These findings are consistent with the presence of HTLV proviral sequences in DNA of a fraction of the primary tumour cells and/or the presence of only fractions of the provirus in most tumour cells.

Thus, the present data indicate that an isolate of a type C retrovirus (HTLV_{MB}) from the cultured leukaemic cells of a patient with Sézary syndrome is closely related to HTLV_{CR} from a patient with T-cell lymphoma, by virtue of their structural protein and nucleic acid sequence homology. Further studies are required to identify any differences between the two isolates. The observations that the HTLV isolates are not ubiquitous genetically transmitted viruses of man⁵, that HTLV-related nucleic acid sequences and proteins are found in fresh leukaemic blood cells of patient M.B. and that specific antibodies to HTLV occur in some human sera^{16,17} indicate that HTLV has infected some humans. The results also suggest that its provirus was integrated into the human genome of at least some of the leukaemic cells. Consistent with these findings, recent electron microscopic studies of van der Loo *et al.* show apparent retrovirus-like particles in primary (uncultured) tumour cells of a patient with cutaneous T-cell lymphoma¹⁸.

Many animal retroviruses are known to cause leukaemias and lymphomas of T cells in their host¹⁹. Particularly interestingly, a cutaneous lymphoma can occur in sheep after inoculation with BLV^{20,21}. HTLV has so far been found only in the neoplastic tissue of three patients with T-cell malignancies. Seroepidemiological studies and surveys of cell nucleic acids for HTLV sequences are in progress to obtain further evidence for a possible relationship of HTLV particles to these diseases.

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Antibodies in human sera reactive against an internal structural protein of human T-cell lymphoma virus

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Although retroviruses (RNA tumour viruses) have been implicated in the causation of naturally occurring leukaemias and lymphomas of animals¹, it is not yet clear whether they are involved in the human versions of these malignant diseases, particularly because of the difficulty in isolating viruses truly ascribable to human origin^{2,3}. However, a novel type C retrovirus (called HTLV_{CR}) has been isolated in our laboratory from T cells (fresh and in culture) from a lymph node biopsy of a patient (C.R.) with cutaneous T-cell lymphoma (mycosis fungoides)⁴ and a very similar virus (HTLV_{MB}) from the peripheral blood T cells of another patient (M.B.) with cutaneous T-cell leukaemia (Sézary syndrome, see accompanying report⁵). The nucleic acid sequence⁶, the reverse transcriptase⁷ and the major internal structural protein (p24)⁸ of HTLV_{CR} are not significantly related to any of the known retroviruses; nucleic acid sequences and p24 protein of HTLV_{MB} have been recognized in fresh and cultured cells⁵. We now describe the results of a limited survey of the occurrence, in patients with T-cell malignancies (and among normal people), of antibodies against HTLV_{CR} proteins. We find that antibodies against p24 are present in human sera (including those of patient C.R. and his wife), and that these are specifically directed at HTLV_{CR} proteins and not at cell-specific determinants—in other words, the immunological reactions are not those reported in human sera^{9–11} against animal virus glycoproteins which, lacking virus specificity, are directed against the carbohydrate residues of the glycoprotein^{12,13}. The antibodies against HTLV are thus the first evidence for a specific immune response in humans against a retrovirus.

A limited survey was designed to determine whether natural antibodies reactive against the human retrovirus isolate, HTLV, can be detected in human sera. The assay used in this preliminary survey was a radioimmune precipitation of ¹²⁵I-labelled HTLV_{CR} p24 by human sera using a double antibody system⁸. The p24 of HTLV_{CR} was purified to homogeneity from density-

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Table 1 Specificity of precipitation of HTLV_{CR} p24 by natural antibodies in human sera

¹²⁵ I-labelled antigen used in RIA	Serum	Diagnosis	Per cent competition by							
			HTLV _{CR}	R-MuLV	FeLV	SSV	BaEV	MPMV	SMRV	BLV
HTLV _{CR} p24	Patient C.R.	Cutaneous T-cell lymphoma	98	3	6	4	3	5	0	2
	Patient M.J.	Cutaneous T-cell leukaemia	93	2	0	0	1	0	3	0
	Wife of patient C.R.	Normal	90	0	1	4	9	5	9	2
R-MuLV gp70	M.R.G.	Normal	100	100	100	100	97	100	100	100
	Patient S-924	Chronic myelogenous leukaemia	72	88	83	80	68	78	79	84

Competition radioimmunoassay (RIA) was performed as described in Fig. 2 legend using the competing viruses at 10 µg protein. Buffer 1 was used in the precipitations of ¹²⁵I-labelled HTLV_{CR} p24 and buffer B in the precipitations of ¹²⁵I-labelled R-MuLV gp70. FeLV, feline leukaemia virus, SSV, simian sarcoma virus, BaEV, baboon endogenous virus; MPMV, Mason-Pfizer monkey virus; SMRV, squirrel monkey retrovirus; BLV, bovine leukaemia virus.

banded virus and radiolabelled with ¹²⁵I as described elsewhere⁸. The serum from patient C.R. precipitated >90% of the labelled p24, and the serum of another patient (M.J.) with Sézary syndrome showed a similar high reactivity towards HTLV_{CR} p24. Twenty-one other serum samples from various T-cell malignancies and 50 sera from random normal donors showed no significant precipitating activity. Sera were also obtained from 11 close family members of patients with T-cell malignancies, with previous evidence of HTLV infection. Out of these, one serum (no. 81-5, from the wife of patient C.R.) reacted strongly in the immunoprecipitation.

The reactivity of the positive sera was characterized in detail, and Fig. 1 shows the pattern of precipitation obtained. The radiolabelled probe used was 95% immunoprecipitable with a hyperimmune rabbit serum against disrupted HTLV_{CR}, whereas sera 38-7 and 24-1 from patients C.R. and M.J. respectively precipitated >80% and the apparently normal serum 81-5 precipitated >50% of the ¹²⁵I-labelled p24 at the highest serum concentration tested. Thus, the two highly positive human sera had antibody titres very similar to that of the hyperimmune rabbit serum. The profile of normal serum M.R.G. in Fig. 1 is typical of the results obtained with all the negative sera tested. These results concur with data obtained in an independent study using solid phase radioimmunoassay which showed specific reactivities in these sera towards HTLV_{CR} (ref. 14).

In view of the past controversy¹⁵ concerning the presence of natural antibodies in human sera reactive with antigens from animal retroviruses, the reactivities shown in Fig. 1 were analysed for their specificity. Various retroviruses and cell

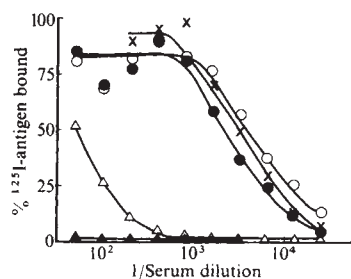


Fig. 1 Immunoprecipitation of ¹²⁵I-labelled HTLV_{CR} p24 by different human sera. HTLV_{CR} p24 was purified and labelled with ¹²⁵I as described elsewhere⁸. The labelled p24 (8,000–10,000 c.p.m.) was mixed with two-fold serial dilutions of human sera or a hyperimmune rabbit serum raised against HTLV in a volume of 20 µl of buffer 1 (200 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.3% Triton X-100, 0.1 mM phenylmethylsulphonyl fluoride and 2 mg ml⁻¹ bovine serum albumin). The reaction mixture was incubated for 2 h at 37 °C and overnight at 4 °C. A 20-fold excess of goat anti-human IgG (or goat anti-rabbit IgG when rabbit IgG was the primary antibody) was then added and the volume made up to 500 µl with buffer 1. The samples were incubated for 1 h at 37 °C and an additional 2 h at 4 °C and then centrifuged at 2,500 r.p.m. for 15 min in a Beckman centrifuge. The supernatants were aspirated and the radioactivity in the pellets was counted in an LKB Ultragamma counter. ●, Human serum (patient C.R.); ○, human serum (patient M.J.); △, human serum (wife of patient C.R.); ▲, normal human serum, M.R.G.; ×, rabbit antiserum to HTLV_{CR}.

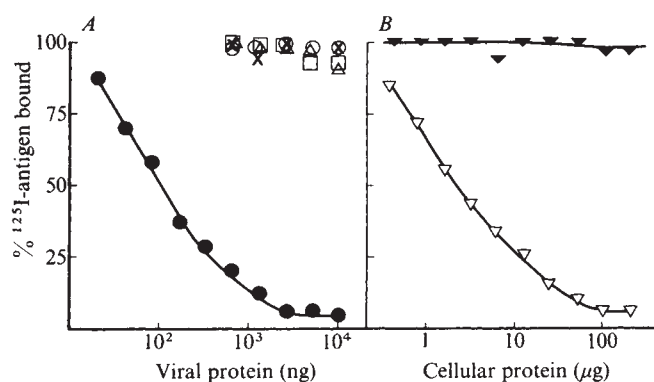


Fig. 2 Specificity of the precipitation of HTLV_{CR} p24 by serum of patient M.J. Competition radioimmunoassays were set up using ¹²⁵I-labelled HTLV_{CR} p24 and a limiting dilution (1:7,500) of the human serum (patient M.J.). Serial dilutions of the unlabelled antigens were preincubated with the serum for 1 h at 37 °C. Labelled p24 (8,000–10,000 c.p.m.) was then added and the reaction mixture incubated and processed as described in Fig. 1 legend. a, Competition by retroviral extracts: ●, HTLV_{CR}; ○, SSV; ×, BaEV; △, SMRV; □, BLV. b, Competition by cell extracts: ▽, HUT102 cells producing HTLV_{CR}; ▼, HUT78 cells.

extracts were tested for their capacity to compete with the precipitation of ¹²⁵I-labelled p24 induced by the positive human sera. Figure 2 shows results obtained with the serum of patient M.J. Simian sarcoma virus, baboon endogenous virus, Mason-Pfizer monkey virus and bovine leukaemia virus failed to neutralize the reactivity of the serum for p24. Only HTLV_{CR} effectively competed in the precipitation (Fig. 2a). Similarly, extracts from cells which produce HTLV_{CR} totally competed in the precipitation, whereas extracts from a neoplastic human T-cell line (HUT78) which is negative for HTLV_{CR} (ref. 8) failed to compete (Fig. 2b). This specificity is very similar to that observed in the precipitation of HTLV_{CR} p24 by a hyperimmune serum against disrupted HTLV_{CR} (ref. 8). The same strict specificity was observed with all the human sera which showed an immune reactivity with HTLV_{CR} p24 (Table 1). Similarly, identical results were obtained whether the immune complex was precipitated with goat anti-human IgG or by the addition of inactivated *Staphylococcus aureus* cells, indicating that the reactive species in the human sera are intact immunoglobulin molecules.

The extent of immune precipitation of ¹²⁵I-labelled p24 by the human sera was not affected by the assay conditions used, unlike the situation encountered with human natural antibodies reported to be reactive to envelope glycoproteins of animal retroviruses (refs 12, 13 and data below). The extent and pattern of precipitation of HTLV_{CR} p24 by human sera were identical, irrespective of whether the immune precipitation medium was supplemented with ovalbumin or bovine serum albumin as a protein carrier (Fig. 3a,b). In contrast, many human sera exhibit strong precipitating activity towards Rauscher murine leu-

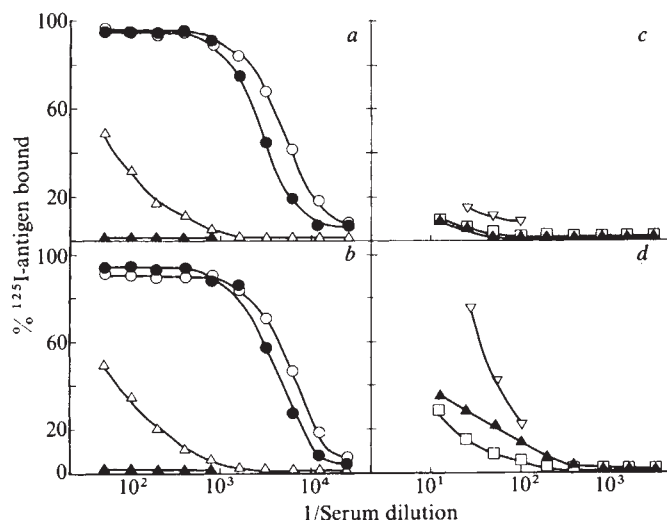


Fig. 3 Effect of buffer composition on the precipitation of ^{125}I -labelled HTLV_{CR} p24 and ^{125}I -labelled R-MuLV gp70 by human sera. Immuno-precipitation of ^{125}I -labelled HTLV_{CR} p24 and ^{125}I -labelled R-MuLV gp70 by the human sera was performed in buffer A (buffer 1 containing 5 mg ml⁻¹ bovine serum albumin and 0.5% Triton X-100) or in buffer B (buffer A containing 5 mg ml⁻¹ ovalbumin instead of bovine serum albumin). Labelled antigen (8,000–10,000 c.p.m.) was incubated with twofold serial dilutions of the human sera in 200 μl of the appropriate buffer and the extent of immune precipitation measured as described in Fig. 1 legend. *a, b* Represent precipitation of HTLV_{CR} p24 in buffer A and buffer B respectively. ●, Serum of patient C.R.; ○, serum of patient M.J.; △, serum of the wife of patient C.R.; ▲, normal serum. *c, d* Represent precipitation of R-MuLV gp70 in buffer A and buffer B, respectively. ▽, serum of chronic myelogenous leukaemia patient, 7-117; □, serum of patient with chronic myelogenous leukaemia, S-964; ▲—▲, normal human serum, M.R.G.

kaemia virus (R-MuLV) gp70 when the reaction is done in the presence of ovalbumin as the carrier (Fig. 3d). This activity is nearly eliminated if bovine serum albumin is used instead of ovalbumin (Fig. 3c). Such reactivity is also observed with gp70s of other retroviruses and is thought to be directed, not against the polypeptide portions of the gp70 that are virus coded, but against the carbohydrate moieties on the molecule that are introduced by the host cell as a post-transcriptional event, and which are therefore cell-specific and not virus-specific^{12,13}. This lack of viral specificity is clearly shown by the finding that, unlike the activity towards HTLV_{CR} p24, all the viruses tested competed totally in the precipitation of R-MuLV gp70 (Table 1).

We have described here a specific immune reactivity in some human sera towards the internal structural antigen p24 of HTLV_{CR}, a type C retrovirus, isolated from the T cells of two different patients with T-cell malignancies. Of 84 sera tested, 3 have shown significant reactivity towards this viral antigen. It is possible that other sera contain anti-HTLV_{CR} antibodies but were missed because the assay detects only antibodies to the HTLV_{CR} core protein, p24. In this regard, use of an envelope antigen of the virus as the probe may show a more representative frequency of natural antibodies in humans towards the virus. These and independent tests using solid phase radio-immunoassay (ref. 14) are the first evidence of a specific immune response to a type C retrovirus in humans. As animal retroviruses can cause T-cell leukaemias and lymphomas, the finding of these antibodies only in sera of people with T-cell neoplasias and in the positive normal case (the wife of the patient from whom HTLV was first isolated), combined with other recent evidence for the presence of this virus in some of these patients, warrants careful consideration of a possible role for HTLV in the origin of these kinds of human leukaemias and lymphomas.

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The *v-sis* transforming gene of simian sarcoma virus is a new *onc* gene of primate origin

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The defective transforming simian sarcoma virus (SSV) and its nondefective helper virus (SSAV) are retroviruses isolated from a fibrosarcoma of a pet woolly monkey. Together with the gibbon ape leukaemia viruses, they are the only group of retroviruses known to cause spontaneous and experimentally induced neoplasia in primates (see refs 1 and 2 for review). Molecular cloning has shown that SSV contains a 1.2-kilobase (kb) transformation-specific viral *onc* gene (*v-sis*)^{3,4} which, like other viral *onc* genes, is derived from a set of conserved cellular DNA sequences⁵. A human DNA fragment containing sequences homologous to the entire *v-sis* gene has also been cloned and analysed⁶. We present here experiments carried out on the cloned SSV genome which show that: (1) *v-sis* is distinct from other viral transforming (*onc*) genes; and (2) *v-sis* is derived from a woolly monkey naturally infected once with gibbon ape leukaemia virus (GaLV) and is to date the only *onc* gene of primate origin.

Different viral *onc* genes have been known to share a common cellular progenitor. There are many examples of viruses isolated from the same species acquiring the same *onc* gene, and at least one example of a cellular *onc* gene represented in viruses from two different species (chicken and cat)⁷. To test possible homology between *v-sis* and other viral *onc* genes, we digested DNA from a recombinant phage clone (C60) of SSV with the restriction endonucleases *Bgl*II or a combination of *Sal*I and *Pvu*II to localize the *v-sis* sequences on a gel, which we blot-hybridized⁸ to ³²P-labelled plasmid clones containing *onc* sequences from different acutely transforming retroviruses. Table 1 summarizes the origin and properties of the probes used. The detailed restriction map of C60 has been published elsewhere³. This clone has two copies of the long terminal repeat (LTR) and an inversion involving one LTR and 0.1 kb of adjacent viral sequences. In each case *Bgl*II or *Sal*I and *Pvu*II generated a 0.65-kb fragment that was highly enriched in *v-sis* sequences, as demonstrated by the poor detection of this

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Table 1 Origin and properties of probes

Viral origin	Ref.	pBR322 sequences	<i>onc</i> sequences (kb)	% Of complete <i>onc</i> genes	Helper sequences (kb)
SSV	3	+	1.2	100	4.8
SSAV	3	+	0	ND	9.0
HaMSV	14	+	1.0	100	5.2
KiMSV	15	+	?	100	?
M-MSV	16	—	0.8	60	0
A-MuLV	17	+	2.5	60	0
FeSV _{ST}	18	+	1.2	85	0
ASV	19	+	1.5	100	8.5
MC29	20	+	2.0	100	0.8
AMV	21	+	0.6	100	1.9

SSAV, simian sarcoma associated virus; HaMSV, Harvey murine sarcoma virus; KiMSV, Kirsten murine sarcoma virus; M-MSV, Moloney murine sarcoma virus; A-MuLV, Abelson murine leukaemia virus; FeSV_{ST}, Snyder-Theilen strain of feline sarcoma virus; ASV, avian sarcoma virus, Schmidt-Ruppin A strain; MC29, avian myelocytomatosis virus; AMV, avian myeloblastosis virus. ND, not determined.

fragment by the SSAV probe compared with the homologous SSV probe (Fig. 1a, b; and see simplified map of C60). None of the other viral *onc* probes detected the 0.65-kb fragment. Except for Harvey murine sarcoma virus (HaMSV) and Kirsten murine sarcoma virus (KiMSV), the murine or feline probes contained no helper sequences and did not hybridize detectably to any SSV DNA fragment (Fig. 1d). Although the avian viral probes did contain helper sequences, they showed no detectable homology with SSAV-derived sequences (Fig. 1d). The HaMSV and KiMSV plasmids used contained the entire viral genome, and hybridization to the 2.8-kb *Pvu*II fragment or 3.3-kb *Bgl*II fragment of SSV (Fig. 1c) was probably due to cross-reactivity of the LTR sequences of the two viruses.

To ensure that the *onc* genes of HaMSV and KiMSV were indeed unrelated to any portion, especially the 3' region, of *v-sis*, further restriction enzyme analyses were carried out. For these experiments, a clone of SSV (C14) which contained one LTR and no inversion in its genome was used²⁴. DNA from C14

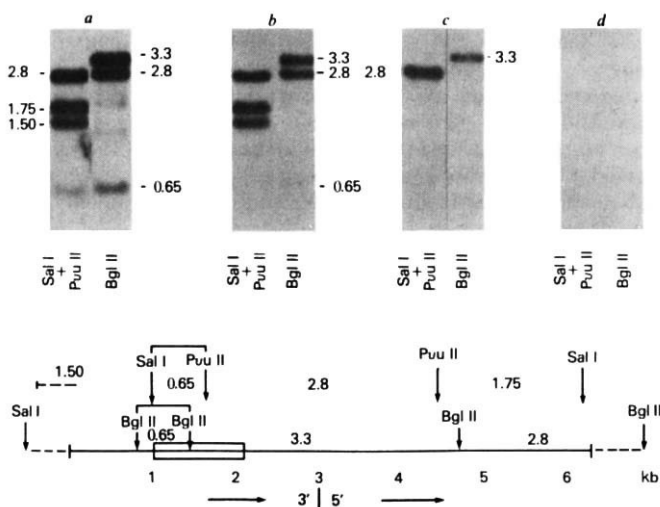


Fig. 1 Lack of homology of *v-sis* to other viral *onc* sequences. DNA (40 ng) from a Charon 21A-SSV recombinant phage (C60) was digested with *Bgl*II or *Sal*I + *Pvu*II on 1.0% agarose gels and transferred to nitrocellulose filters as described elsewhere⁹. Multiple replicate filters were made and each hybridized to a ³²P probe prepared from the cloned DNA after nick translation²³. a, SSV; b, SSAV; c, HaMSV, KiMSV; d, M-MSV_{onc}, FeSV_{onc}, A-MuLV_{onc}, AMV, MC29, ASV(SR). The filters were washed in 1 × SSC at 60 °C and autoradiography was carried out for 4–16 h. A simplified map of C60 is shown at the bottom. Sizes are given in kb.

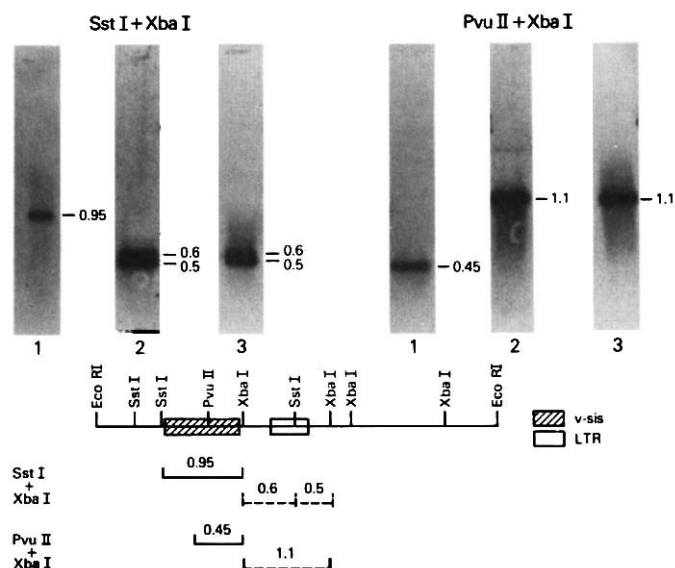


Fig. 2 Assay for homology between a Charon 21A-SSV recombinant phage (C14) and plasmid clones of HaMSV and KiMSV. Triplicate filters containing C14 DNA digested with *Sst*I + *Xba*I or *Pvu*II + *Xba*I were hybridized to ³²P-labelled probes of: (1) p335, a plasmid subclone of the 3' half of a human *c-sis* gene, (2) HaMSV and (3) KiMSV. A simplified map of C14 indicating the *v-sis* and LTR sequences is also shown. Sizes are given in kb.

was co-digested with *Sst*I and *Xba*I or with *Pvu*II and *Xba*I. These digestions delimited more precisely the boundaries of *v-sis* and LTR sequences (see map in Fig. 2). To locate more conveniently the 3' region of *v-sis*, a subclone (p335) derived from a *Bam*HI-*Bam*HI fragment of the human *c-sis* gene⁶ homologous to the 3' two-thirds of *v-sis* was used as a probe. This clone has no SSAV-related sequences⁶. Replicate filters were hybridized to ³²P-labelled pBR322 plasmid clones containing HaMSV and KiMSV genomes. p335 detected a 0.95-kb fragment in the *Sst*I + *Xba*I digestion and a 0.45-kb fragment in the *Pvu*II + *Xba*I fragment (Fig. 2), as expected from the map. The HaMSV and KiMSV probes, in contrast, hybridized only to fragments containing LTR sequences (0.6 and 0.5 kb in the *Sst*I + *Xba*I digest and 1.1 kb in the *Pvu*II + *Xba*I digest) and not at all to *sis*-containing fragments. This lack of homology between *v-sis* and *v-Harvey-ras* is consistent with our previous demonstration that *v-sis* and *v-Harvey-ras* hybridized to distinct sets of sequences in vertebrate DNA⁵. Here we also show that *v-sis* is distinct from *v-Kirsten-ras*. Thus, taken together, our results suggested that *v-sis* was distinct from other viral *onc* genes. Of particular interest was the lack of homology between *v-sis* and the M-MSV, HaMSV or KiMSV *onc* genes. The genetic structure of SSV is most similar to M-MSV in that its gene order is 5'- Δ *gag*- Δ *env*-*onc*-C-3' (ref. 3). Its transformation-specific protein product is a 20,000-molecular weight protein (our unpublished data, and J. Thiel and D. Bolognesi, personal communication), similar in size to the transforming protein (p21) of KiMSV and HaMSV⁹. Our results suggest that *v-sis* is distinct from these three rodent-derived *onc* genes.

All the known viral *onc* genes are derived from normal host cell sequences that are phylogenetically conserved among vertebrates. Using a non-stringent and sensitive hybridization technique such as Southern hybridization⁸, we have shown that *v-sis* sequences could detect homologous loci in DNA from chicken to man⁵. Determination of its actual host species of origin, however, would require a more stringent assay of homology. We have subcloned a *Pst*I-*Pst*I fragment of the SSV genome that contains ~250 base pairs (bp) of 5' *v-sis* sequences and 900 bp of SSAV-derived sequences into the bacteriophage M13. The single-stranded phage DNA was then partially digested with DNase and terminally labelled with ³²P by the kinase

Table 2 Species of origin of *v-sis* sequences

	S_1 nuclease resistant (c.p.m.)	% <i>v-sis</i> sequences hybridized
Woolly monkey	11,000	87
Marmoset	9,200	71
Gibbon	6,100	54
Human	5,500	42
Rat	1,000	8
Cat	800	6

The M13 mp7 recombinant phage was constructed as described previously²². The single-stranded phage DNA was purified, 5 μ g of the DNA were digested with 0.4 ng DNase I for 15 min at 25 °C, extracted with phenol and dialysed. The partially digested DNA was then treated with bacterial alkaline phosphatase (3 U) at 60 °C for 30 min, extracted with phenol and precipitated in ethanol. The precipitate was collected by centrifugation, and incubated with polynucleotide kinase and 0.2 mCi [γ -³²P]ATP (7,000 Ci nmol⁻¹) at 37 °C for 30 min. The labelled DNA was purified by three cycles of ethanol precipitation in ammonium acetate buffer. About 400,000 c.p.m. of the labelled probe (~12,000 c.p.m. of *v-sis* sequences) was hybridized to 500 μ g of cellular DNA to a C_{ot} of $>10^4$. Hybridization was monitored by S_1 nuclease resistance as described elsewhere¹.

reaction¹⁰. The labelled DNA was hybridized in solution to cellular DNA from tissues of different animals and the resultant hybrids assayed by their resistance to S_1 nuclease¹¹. Of all the DNA examined, primate DNA hybridized significantly better than DNA from non-primates (Table 2). Among the primates, the New World monkeys (woolly monkey and marmoset) contained more DNA sequences homologous to *v-sis* than the Old World apes (gibbon and man) and the highest homology was obtained with woolly monkey DNA. The SSAV genome had no detectable homology to primate DNA either by Southern hybridization^{5,6} or liquid hybridization (not shown), and its distant homology to rodent DNA could only be monitored by non-stringent hybridization conditions¹². We conclude that *v-sis* originated from woolly monkey DNA. The close relatedness of SSAV to different GaLV isolates and the revelation that the woolly monkey that yielded SSV/SSAV co-habited with a gibbon before it developed sarcoma¹¹ suggest that SSAV was transmitted from the gibbon to the woolly monkey during that period, and that in a single infection event, SSAV had recovered an *onc* gene from woolly monkey to generate SSV.

Although all viral *onc* genes thus far identified are conserved among vertebrate species from bird to man, there seems to be some species specificity with respect to the particular *onc* gene(s) accessible for recombination. For example, the same chicken *onc* gene (*myc*) was recovered in four independent recombination events to generate the leukaemia viruses MC29, MH2, CMII and OK10 (ref. 13). The only known example of related cellular *onc* genes from different species becoming viral *onc* genes is the relationship between the feline sarcoma virus (Snyder-Theilen and Gardner-Arnstein strains) and avian Fujinami sarcoma virus⁷. Therefore, the genetic arrangement of a particular *onc* gene may vary between species and may determine its frequency or recombination. As *v-sis* is the first known *onc* gene derived from a primate, it would be of interest to see whether subsequent primate virus isolates will pick up the same gene.

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Structure of the glycoprotein gene in rabies virus

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Rabies virus, a rhabdovirus, has a single (non-segmented) negative-strand RNA genome which is transcribed on infection to produce five polyadenylated complementary monocistronic mRNA species. Each of the virus-specific mRNAs representing a structural gene of the rabies virus genome¹ codes for a virion structural protein which corresponds in size to the apparent coding capacity of its mRNA^{2,3}. The glycoprotein gene codes for a membrane-associated molecule which forms spike-like projections on the surface of mature rabies virions and is responsible for the induction and binding of virus-neutralizing antibodies to the virus^{4–6}. To define the antigenic and immunogenic properties of rabies virus glycoprotein, we have cloned a cDNA copy of its mRNA sequence into pBR322 (ref. 7). Here we describe the characterization of the cDNA sequence, which has allowed us to predict several features of the glycoprotein from the deduced amino acid sequence.

The five rabies virus-specific mRNAs for L, G, N, M₁ and M₂ proteins have been characterized by sucrose gradient³ and gel analysis²: L mRNA sediments at $>28S$, glycoprotein (G) mRNA at 18S, nucleocapsid (N) mRNA at 16S and matrix (M₁ and M₂) mRNAs at 12S. For the cloning of the G mRNA, RNA extracted from rabies virus-infected BHK cells was purified by oligo(dT) cellulose chromatography and sucrose density centrifugation. Poly(A) RNA of ~15–20S was used as starting material (thus eliminating the mRNAs for L, M₁ and M₂ proteins) for the synthesis of double-stranded complementary DNA. Synthesized DNA was inserted into pBR322 at the *Pst*I site by dG–dC tailing (see Fig. 1 legend). Approximately 1% of the tetracycline-resistant colonies screened using ³²P-labelled rabies virion RNA responded with varying intensities. From 100 colonies that were re-screened, the 20 giving the strongest signals were selected. The size of the plasmids was checked by digestion with *Bam*HI to give linear molecules and with *Pst*I to excise the insert. About half the plasmids contained an additional *Bam*HI site (group A) while the others contained a single *Bam*HI site (group B). This suggested the presence of two different sequences, which we presumed coded for glycoprotein and nucleocapsid protein.

To distinguish between the putative glycoprotein and nucleocapsid protein mRNA-specific plasmids, the insert of a plasmid from each group was isolated by *Pst*I digestion and

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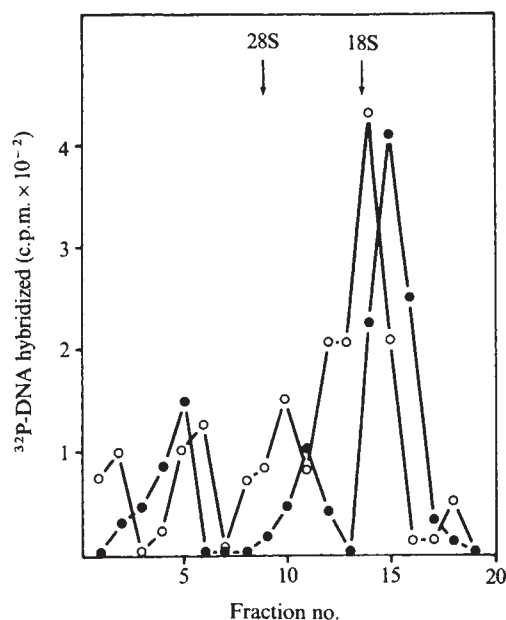


Fig. 1 Hybridization analysis of rabies virus-infected cell poly(A) RNA with rabies recombinant plasmids. Plasmids were constructed as follows: 15–20S poly(A) RNA from rabies virus (ERA strain)-infected cells was transcribed into cDNA using oligo(dT) as primer and AMV reverse transcriptase (supplied by J. Beard), followed by synthesis of double-stranded (ds) cDNA with *Escherichia coli* DNA polymerase¹⁷. After S_1 nuclease treatment, ds-cDNA was elongated with dCMP residues by deoxynucleotidyl terminal transferase, and ds-cDNA >1 kbp was selected by sucrose density centrifugation. ds-cDNA tailed with dCMP was hybridized to pBR322 cut with *Pst*I and tailed with dGMP residues, and the recombinant plasmid used to transform *E. coli* 1776 (ref. 18). Colonies containing rabies virus-specific sequences were selected by colony hybridization¹⁹ using rabies virion RNA labelled with ^{32}P by polynucleotide kinase after partial alkali hydrolysis. For hybridization analysis, poly(A) RNA (~130 μg) was heat-denatured at 100 °C for 1 min and centrifuged in a 5–23% sucrose gradient containing 50 mM Tris-HCl pH 7.5, 5 mM EDTA at 25,000 r.p.m. at 10 °C for 16 h in a Beckman SW41 rotor. Each fraction (0.5 ml) from the gradient was diluted 1:60 with water and 1 μl was hybridized with labelled insert (~3,000 c.p.m., specific activity 10^8 c.p.m. μg^{-1}) prepared from plasmids A and B. Before hybridization, the probes were heated at 100 °C for 5 min, adjusted to 50% formamide, 0.75 M NaCl, 10 mM HEPES buffer (pH 6.8), 1 mM EDTA, 1 mg ml^{-1} yeast RNA, and heated with fractionated poly(A) RNA from rabies virus-infected cells in a total volume of 10 μl at 45 °C for 16 h in paraffin oil. Hybridization was stopped by addition of cold 0.25 M NaCl, 0.03 M sodium acetate (pH 4.5), 1 mM ZnCl_2 . After removal of paraffin oil, S_1 nuclease (100 units) was added and the solution incubated at 37 °C for 45 min. Acid-insoluble counts were determined by trichloroacetic acid precipitation. O, B; ●, A.

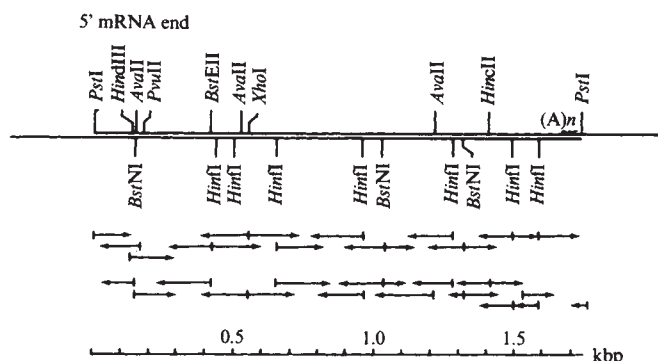


Fig. 2 Restriction map of the insert of pRG. The map was constructed from the size of DNA fragments arising from digestion with combinations of restriction enzymes and estimated by agarose or acrylamide gel electrophoresis¹⁰. The upper set of arrows indicates regions and directions sequenced from the cDNA strand. At the end of the cDNA sequence corresponding to the 5' terminus of the mRNA, there is an oligo(dG-dC) tract of 24 residues with an oligo(dG-dC) tract of 14 residues at the opposite end. Also present at this end is a sequence of poly(dA) which exists in our clone as two lengths of ~50 and 80 bp.

electrophoresis on 1.5% agarose gel⁸. Each insert was labelled by nick-translation⁹ and hybridized to individual fractions of poly(A) RNA derived from rabies virus-infected cells and sedimented in a sucrose gradient (Fig. 1). The insert of a group B plasmid hybridized to a mRNA sedimenting at 18S, the location of maximal mRNA activity for the synthesis of glycoprotein, while the insert of group A hybridized to a mRNA sedimenting at 16S, the location of mRNA capable of directing the synthesis in oocytes of nucleocapsid protein (see Fig. 1 in ref. 3). Thus the plasmids of groups A and B were designated pRN and pRG respectively.

Restriction mapping established the size of the inserted DNA of pRG to be ~1.75 kilobase pairs (kbp; Fig. 2). To determine the number of nucleotides which might be missing from the glycoprotein cDNA insert, a small labelled fragment located close to the 5' end of the glycoprotein sequence and bounded by restriction sites for *Pvu*II and *Hind*III was hybridized to 18S poly(A) RNA from rabies virus-infected cells. Incubation of the hybrid formed between glycoprotein mRNA and labelled DNA

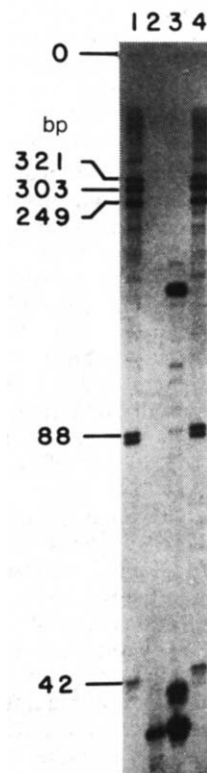


Fig. 3 Comparison of lengths of the glycoprotein cDNA clone and its mRNA. The glycoprotein gene sequence was restricted with *Pvu*II, labelled by [γ - ^{32}P]ATP with polynucleotide kinase, and subsequently digested with *Hind*III. The 5'-labelled 36-bp fragment located close to the 5' terminus of the glycoprotein sequence was isolated on a 5% polyacrylamide (0.1% bisacrylamide) gel in 50 mM Tris-borate pH 8.3, 1 mM EDTA, eluted and dried. The ^{32}P -labelled fragment was then denatured in 20 μl of 80% (v/v) formamide by heating at 100 °C for 3 min. A sample of 18S poly(A) RNA (10 μg) from rabies virus-infected cells was dried down with 20 μl of solution containing 0.4 M NaCl, 20 mM HEPES buffer pH 6.8, and 1 mM EDTA pH 7.5. The solution of denatured ^{32}P -labelled DNA was added to the dried RNA and incubated at 60, 58, 56 and 54 °C for 1 h at each temperature. After annealing, the mixture was transferred to 100 μl cold 0.3 M sodium acetate pH 5.5 and precipitated by 2.5 vol ethanol at -20 °C. The precipitated nucleic acids were dissolved in 40 μl of 50 mM Tris-HCl pH 8.3, 10 mM MgCl_2 , 35 mM KCl, 30 mM β -mercaptoethanol, 0.5 mM each of the four deoxynucleoside triphosphates, 200 μg ml^{-1} bovine serum albumin, 12.5 μg ml^{-1} actinomycin D and AMV reverse transcriptase (12 U), and incubated at 37 °C for 90 min. After precipitation from the reaction mixture with ethanol, the nucleic acids were dissolved in 4 μl 80% formamide, 50 mM Tris-borate pH 8.3, 1 mM EDTA, 0.1% xylene cyanol FF and 0.1% Bromophenol blue and heated at 90 °C for 1 min before loading on to a 8% polyacrylamide (0.4% bisacrylamide) gel containing 50% (w/v) urea, 100 mM Tris-borate pH 8.3, 2 mM EDTA. Labelled nucleic acids were detected by autoradiography using an intensifying screen at -70 °C. Lanes 1 and 4, labelled size markers; lane 2, labelled 36-bp fragment bounded by *Pvu*II and *Hind*III; lane 3, labelled 36-bp fragment elongated by AMV reverse transcriptase. O, origin.

Fig. 4 DNA sequence of the mRNA sense strand and the deduced amino acid sequence for rabies virus glycoprotein (ERA strain). Numbers in parentheses refer to the amino acids counted from the N-terminal lysine residue of the mature glycoprotein located 20 amino acids from the presumed initiation codon. A shaded hydrophobic domain close to the C-terminal end is presumed to be the transmembrane segment. Asterisks mark three possible carbohydrate attachment sites based on the presence of Asn-X-Ser and Asn-X-Thr as described in the text. Boxes show the initiation codon (ATG) and termination codon (TGA).

AGGAAG

ATG GTT CCI CAG GCT CTC CTG TTT GTA CCC CTT CTG GTT TTT CCA TTG TGT TTT GGG AAA
met val pro gln ala leu leu phe val pro leu leu val phe pro leu cys phe gly lys
(1)

TTC CCI ATT TAC ACG ATA CTA GAC CTT GGT CCC TGC AGC CCG ATT GAC ATA CAT CAC
phe pro ile tyr thr ile leu asp lys leu gly pro trp ser pro ile asp ile his
(10)

CTC AGC TGC CCA AAC AAT TTG GTA GTG GAG GAC GAA GCA TGC ACC AAC CTG TCA GGG TTC
leu ser cys pro asn asn leu val val gly asp gly gly cys thr asn leu ser gly phe
(30)

TGC TAT ATG GAA CTT AAA GTT GCA TAC ATC TTA GGC ATA AAA ATG AAC GGG TTC ACT TGC
ser tyr met gln leu lys val gly tyr ile leu lys met asn gly phe thr cys
(50)

ACA GGC GTT GTC ACG GAG GCT GAA ACC TAC ACT AAC TTC GTT GGT TAT GTC ACA ACC ACG
thr gly val val thr glu ala glu thr thr thr asn phe val gly tyr val thr
(80)

TTT AAA AAG AAG CAT TTC CCG CCA ACA CCA GAT GCA TGT AGA GCG GCG TAC AAC TGG AAG
phe lys arg lys his phe arg pro thr pro asp ala cys arg ala ala tyr asn trp lys
(90)

ATG ACC GGT GAC CCC AAG TAT GAA GAG TCT CTA CAC AAT CCG TAC CCT GAT TAC CCG TGG
met ala gly asp pro arg tyr glu gly ser leu his asn pro tyr pro asp tyr thr
(110)

CTT GCA ACT GTA AAA ACC ACC AAG GAG TCT CTC GTT ATC ATA TCT CCA AGT GTA CCA GAT
leu arg thr val lys thr thr lys glu ser leu val ile ile ser pro ser val ala asp
(130)

TTG GAC CCA TAT GAC AGA TCC CTT CAC TGC AGG GTT TCT CCG ACC GGG AAG TGC TCA GCA
leu asp pro tyr asp arg ser leu his ser arg val phe pro ser gly cys ser gly
(160)

GTA GCG GTG TCT TCT ACC TAC ACT AAC CAC GAT TAC ACC ATT TGC ATG CCC GAG
val ala val ser ser thr tyr cys thr thr asn his asp tyr thr ile trp met pro glu
(180)

AAT CCG AGA CTA GCG ATG TCT TGT GAC ATT TTT ACC AAT AGT AGA GGG AAG AGA CCA TCC
asn pro arg leu gly met ser cys asp ile phe thr asn ser arg gly lys arg ala ser
(200)

AAA GCG AGT GAC ACT TGC GGC TTT GTA ATG GAA AAG CCG CTA TAT AAG TCT TTA AAA GCA
lys arg thr thr cys phe val asp glu arg gly leu thr lys ser leu lys gly
(210)

GCA TGC AAA CTC AAG CTA TGT GCA GTT CTA GCA CTT AAG CTT ATG CAT GCA ACA TGC GTC
ala cys lys leu lys leu cys gly val leu gly leu arg leu met asp gly thr trp val
(230)

ACG ATG GAA CCA TCA AAT GAA ACC AAA TGG TGC CTT CCC GAT CAG TTG GTC AAC CTG CAC
ala met gln thr ser asn glu thr thr lys trp cys pro pro asp gln leu val asn leu his
(250)

GAC TTT GGC TCA GAC GAA ATT GAG CAC CTT GTT GAT GAG GAG TTG GTC AGG AAG ACA GAG
asp phe arg ser asp glu ile glu his leu val leu val glu leu val arg lys arg glu
(270)

GAG TGT CTG GAT GCA CTA GAG TCC ATC ATG ACA ACC AAG TCA GTG AGT TTA AGA GTT CTC
glu cys leu asp ala leu glu ser ile met thr thr lys ser val ser phe arg arg leu
(290)

AET CAT TTA AGA AAA CTT GTC CTT GGG TTT GCA AAA CCA TAT ACC ATA TTC AAC AAG ACC
ser his leu arg lys lys leu val pro gly phe gly lys ala tyr thr ile phe asn lys thr
(310)

TTG ATG GAA CCG GAT GCT CAC TAC AAG TCA GTC AAG ACT TGG AAT GAG ATC CTT CTT TCA
met met glu ala asp ala his thr lys ser val arg thr thr asp asn glu ile leu pro ser
(330)

AAA GGG TGT TTA AGA GTT GGG GGG AGG TGT CAT CCT CAT GTG AAC GGG GTG TTT TTT AAT
lys gly cys leu arg val gly gly arg cys his pro his val asn gly val phe phe asn
(350)

GCT ATA ATA TTA GCA CCT GAC GCG AAT GTC TTA ATC CCA GAG ATG CAA TCA TCC CTC CTC
glu ile ile leu glu ser pro asp gly asp val leu ile pro glu met gln ser thr cys leu
(370)

CAG CAA GAT ATG GAG TGT TTA GAA TGC TGC GTT ATC CCC CTT GTG CAC CCG CTC GCA GAC
gln gln his met glu leu leu glu ser ser val ile pro leu val his pro leu ala asp
(390)

CGG TCT ACC GTT TTT AAG GAC GGT GAC GAG GCT GAG GAT TTT GTT GAA GTT CAC CTT CCC
pro ser thr val phe lys asp gly asp glu ala glu asp phe val glu val his leu pro
(410)

BAT GTG CAC AAT GAT GTC TCA GGA GTT GAC GTT GCT CCG AAC TGG GGG AAG TAT GTA
asp val his asn gln val ser gly val asp leu gly pro asn trp glu thr thr tyr eal
(430)

TTA CTG AET GCA GGG GGC CTC ACT GCG TTG ATG TTG ATA ATT TTC CTG ATG ACA TGT TGT
leu thr met gln ala ggg gcc ctc act gcg ttg atg ttg ata att ttc ctg atg aca tgt tgt
(450)

AGA AGA GTC AAT GAT TCA GAA CCT ACG CAA CAC AAT CTC AAG GGG ACA GGG AAG GAG GTG
arg arg val asn arg ser glu pro thr gln his asn leu arg gly thr gly arg glu val
(470)

TCA GTC ACT CCC CAA ACG GGG AAG ATC ATA TCT TCA TGG GAA TCA CAC AAG AGT GGG GGT
ser val thr pro gln ser gln lys ile thr leu ser trp glu ser his lys ser thr arg
(490)

GAG AAG ACA CTG TCA GGA CTGGCGCTCTTTCAACGATCCAGCTCGAAGATCACCTCCCTTGGGGGGT
glu thr arg leu

fragment with avian myeloblastosis virus (AMV) reverse transcriptase extended the primer to the 5' terminus of the glycoprotein mRNA. Products of the reaction were resolved on a denaturing gel and identified by autoradiography (Fig. 3). Apart from the labelled primer fragment (actual length 36 nucleotides), the major products were ~40 and 165 nucleotides long. The 40-nucleotide band presumably arose by filling in the *Hind*III site recreated by the annealing of *Pvu*II-*Hind*III DNA strands, while the 165-nucleotide band resulted from the extension of the primer which was hybridized to glycoprotein mRNA. As the *Pvu*II site is located 129 bp from the 5' terminus of the cloned glycoprotein sequence (excluding the dG-dC tail of 24 bp), our results indicate that the cloned glycoprotein sequence is missing only ~35 nucleotides from the 5' terminus of the glycoprotein mRNA.

The complete nucleotide sequence of the glycoprotein cDNA (see Fig. 4) was determined by the Maxam and Gilbert technique¹⁰ using both 5'- and 3'-labelled restriction fragments derived from both strands defined by the sites indicated in Fig. 2. From the nucleotide sequence, a polypeptide 524 amino acids long was deduced, beginning with an initiation codon—ATG—located 8–10 nucleotides from the 5' end of the clone. The other two reading frames contained numerous stop codons, such that the longest polypeptide which can be derived from them is <50 amino acids long.

The first six amino acid residues of the mature glycoprotein, NH₂-Lys-Phe-Pro-Ile-Tyr-Thr-, have been identified by direct amino acid sequence analysis of purified rabies virus (ERA strain) glycoprotein, as well as the C-terminal sequence Thr-Arg-Leu (C.-Y. Lai and B. Dietzschold, unpublished results). These sequences are located in the deduced sequence 20–25 and 522–524 residues from the putative initiating methionine. The initial 19 amino acids that precede the N-terminal lysine of the glycoprotein presumably represent a signal peptide as they are predominantly hydrophobic. An uninterrupted hydrophobic domain of 22 amino acids, bounded by lysine 439 (numbered from the N-terminal lysine) and two arginines at positions 462 and 463 near the carboxy-terminal region of the predicted polypeptide, is similar to the proposed transmembrane segment of Semliki Forest virus (SFV) glycoprotein¹¹. A relatively long sequence of 44 charged and uncharged residues extends from the presumptive transmembrane domain to the C-terminal Leu 505 (compare with the viral glycoproteins of SFV¹¹ and

influenza virus¹²). This apparent hydrophilic cytoplasmic domain of the rabies glycoprotein may provide a site for strong interaction with the viral matrix (M_2) and nucleocapsid proteins.

The deduced amino acid sequence contains four carbohydrate acceptor sites as defined by the sequence Asn-X-Ser and Asn-X-Thr; three of these are located on the N-terminal side of the proposed transmembrane segment. Three carbohydrate chains have been found on the rabies virus glycoprotein of the ERA strain¹³, which agrees with the predicted number of carbohydrate attachment sites excluding Asp 465 within the putative cytoplasmic domain.

The absence of the sequence AAUAAA, present in eukaryotic polyadenylated mRNA¹⁴, in the 3'-noncoding region of rabies G mRNA and vesicular stomatitis virus mRNAs¹⁵, presumably reflects the role of virus-associated proteins in polyadenylation of these mRNAs¹⁶ in place of host enzymes.

The cloned cDNA of rabies glycoprotein mRNA was readily identified and distinguished from nucleocapsid protein cDNA clones by (1) size selection of the poly(A) RNA identified by its ability to direct synthesis of glycoprotein in microinjected oocytes³; (2) hybridization with labelled purified rabies virion RNA; and (3) hybridization with glycoprotein mRNA as indicated by the correspondence of the profiles of hybridized RNA and RNA with translational capacity for glycoprotein. The accuracy of the cloned glycoprotein cDNA sequence was confirmed by the agreement between the predicted amino acid sequence and that determined by direct N-terminal and C-terminal amino acid analysis.

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Physicochemical and antigenic properties of synthetic fragments of human leukocyte interferon

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Although interferons (IFNs) were originally described as proteins conferring antiviral resistance to eukaryotic cells¹, various additional biological effects have since been attributed to them, including inhibition of cell proliferation and modulation of the immune response². More recently it was recognized that interferons from different sources have similar amino acid sequences, suggesting their evolutionary relationship. When the sequences of human interferons of the α and β type were aligned to give maximum homology, a largely conserved region was found near the carboxyl terminus of the molecules^{3,4}. The homology of human leukocyte IFN- α_1 and human fibroblast interferon between positions 111 and 166, for instance, was 40%. As conservation of segments of polypeptide chains during evolution may indicate their functional importance⁵, we synthesized three carboxyl-terminal fragments of human IFN- α_1 (ref. 6) ranging in size from 33 to 96 amino acid residues. If the folding of these fragments was similar to that of the corresponding segments in the intact protein, it was conceivable that they had biological activity and that antibodies raised against the synthetic fragments cross-reacted with natural interferon. Such antibodies could be used for the affinity purification of human interferon produced in bacteria and for radioimmunoassays. As we show here, none of the fragments had antiviral activity. A mouse monoclonal antibody prepared against a 56-residue fragment also bound intact IFN- α_1 and IFN- α_2 without neutralizing their antiviral effect. Thus, this antibody, directed against a largely conserved region, may show substantial cross-reactivity within the interferon protein family.

Three carboxyl-terminal fragments of human IFN- α_1 (ref. 6), comprising residues 134–166, 111–166 and 71–166, were synthesized by the solid-phase method^{7–9}. Experimental details are given for the 56-residue fragment because antibodies have previously been raised only against an extensively purified sample of this polypeptide. The sequence of the 56-residue fragment is shown in Fig. 1.

After deprotection and cleavage of the synthetic product from the solid support, the sulphhydryl group of Cys 139 was blocked

by reaction with iodoacetamide. In natural IFN- α_1 this cysteine residue may form a disulphide bond with Cys 29, analogous to IFN- α ¹⁰. The carboxamidomethylated fragment was purified on Sephadex G-50 (Fig. 2) and silica gel 60 and had the following amino acid composition (values expected are in parentheses): CM-Cys 1.0 (1), Asp 3.0 (3), Thr 2.9 (3), Ser 4.5 (5), Glu 5.7 (6), Pro 1.0 (1), Ala 4.1 (4), Val 2.7 (3), Met 2.0 (2), Ile 3.0 (3), Leu 8.0 (8), Tyr 2.8 (3), Phe 1.0 (1), Lys 5.0 (5), Trp 0.9 (1), Arg 6.7 (7). The synthetic 56-residue fragment of IFN- α_1 was homogeneous on TLC in *n*-butanol/acetic acid/pyridine/water (R_F = 0.74) and on thin-layer electrophoresis in 2 M HCOOH, 2 M urea at pH 2.0. Its aromatic region circular dichroism (CD) spectrum and fluorescence emission spectrum were typical of tryptophan-containing polypeptides.

The most striking physicochemical property of the 56-residue interferon fragment was its low solubility above pH 7. Interferon 111–166 has a net charge of +6 whereas the entire IFN- α_1 molecule has a net charge of –3. The natural protein contains 17 aromatic amino acid residues whereas the synthetic fragment contains 5, including 3 of the 4 tyrosines and 1 of the 2 tryptophans. Twenty-six of the 56 residues of the synthetic fragment are hydrophobic, with a calculated hydrophobicity index¹¹ of 31.9, lower than that of insulin (35.7), a protein of similar size. This suggests that the solubility properties of the interferon fragment cannot be explained by its amino acid composition.

The structure of synthetic interferon 111–166 in solution was investigated using CD. Analysis of the far-UV spectrum of a 0.1% solution of the synthetic fragment in 0.01 M phosphate, 0.09 M NaCl, pH 5.0, yielded values of 24 and 36% for α -helix and β -sheet content respectively, using the method of Provencher and Glöckner¹², in contrast to the secondary structure predicted for the corresponding region of IFN- α_1 (65% α -helix and 20% β -sheet)¹³. Interferon 111–166 was stable to urea denaturation up to a denaturant concentration of ~4 M but was unfolded by 8 M urea. The CD spectrum indicated that in the latter condition the molecule approximates a random coil. This process was almost fully reversible (>90%), the native CD spectrum being restored by diluting out the denaturant. This suggests that the fragment is capable of considerable self-organization, producing a structure that, in the light of the immunological results, is probably close to that in the intact interferon molecules.

Polyclonal antisera raised in rabbits or mice against the synthetic 56-residue fragment did not neutralize the antiviral activity of human leukocyte interferon. However, they seemed to bind to interferon, as binding to the fragment (assayed in a solid-phase radioimmunoassay as described in Table 1 legend) was competitively inhibited by *Escherichia coli*-derived leukocyte IFN- α_1 or - α_2 , interferon from human buffy coat lymphocytes and virus-induced mouse interferon, but not by unrelated proteins (data not shown). This suggested that monoclonal

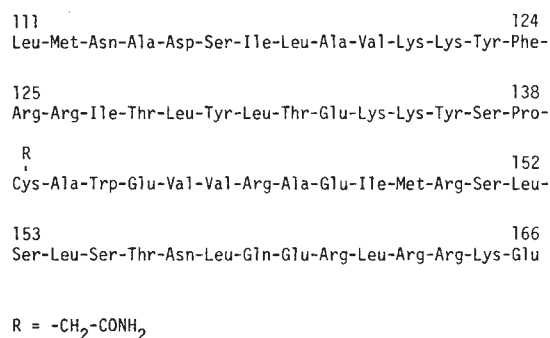


Fig. 1 Amino acid sequence of the synthetic carboxyl terminal 56-residue fragment of IFN- α_1 based on the nucleotide sequence of cloned cDNA⁶.

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Table 1 Binding of monoclonal antibodies directed against the carboxyl-terminal 56-residue fragment of human leukocyte IFN- α_1 to intact IFN- α_1 and - α_2 and to various synthetic IFN- α_1 fragments

Clone	Ig subclass	¹²⁵ I-labelled <i>Staphylococcus aureus</i> protein (c.p.m.) bound to plates coated with					
		Ovalbumin	IFN- α_1	IFN- α_2	IFN- α_1 fragment corresponding to residues		
					71-166	111-166	134-166
I/5	IgG3/ κ	60	106	33	129	1,050	57
I/7	IgG2a/ κ	111	153	71	598	1,201	1,069
II/3	IgG1/ κ	164	169	164	661	1,085	524
II/11	IgG1/ κ	166	177	155	725	1,230	653
II/14	IgG1/ κ	90	181	55	743	1,067	987
II/20	IgG2a/ κ	119	140	66	145	1,233	83
III/17	IgG3/ κ	71	125	71	151	1,059	107
III/21	IgG1/ κ	88	1,090	1,026	1,087	1,004	1,102
VII/8	IgG3/ κ	82	242	200	463	1,135	407
XXI/5	IgG1/ κ	156	163	84	563	1,064	866
XXIV/11	IgG2a/ κ	94	187	87	673	1,271	1,264

The synthetic human IFN- α_1 111-166 was intraperitoneally injected into three BALB/c mice as 50 μ g of protein mixed with complete Freund's adjuvant. A first booster injection with the same dose of antigen in incomplete Freund's adjuvant was given after 3 weeks. A second booster, after another interval of 4 weeks, consisted of 50 μ g of protein per animal injected intraperitoneally with incomplete adjuvant and 50 μ g of protein per animal dissolved in phosphate-buffered saline (PBS) and given intravenously. After 3 days, the spleen cells of the three animals were pooled and 1.5×10^8 cells were fused to 0.75×10^8 cells of the mouse myeloma cell line Fo using polyethylene glycol of molecular weight 4,000 as the fusing agent. Hybrid cells were selected for growth in hypoxanthine/aminopterin/thymidine medium. Supernatant fluids of cell cultures were screened for the presence of specifically binding antibodies in a solid phase radioimmunoassay. The 56-residue interferon fragment was dissolved in PBS at a concentration of 5 μ g ml⁻¹ and was adsorbed to polyvinylchloride microtitre plates. Additional protein binding sites were blocked by a 5-h exposure to 3% solution of ovalbumin. The supernatant fluids were exposed to the wells of microtitre plates for 4 h at room temperature. The presence of fragment-binding immunoglobulins was detected by assaying the radioactivity bound to the wells exposed to a rabbit anti-mouse immunoglobulin serum together with radiolabelled *S. aureus* protein. Cells of initially positive cultures were cloned twice by a limiting dilution technique and injected into female BALB/c mice. Ascitic fluids, diluted 1:1,000, were tested as described above. The 33- and 96-residue fragments of interferon were adsorbed to plates as described for the 56-residue peptide. *E. coli*-derived human leukocyte IFN- α_1 and - α_2 , each at a concentration of 10 μ g ml⁻¹ in PBS and of 10% purity, were similarly adsorbed to the plates. Each value shown represents the mean counts per min from three wells. Activities up to 200 c.p.m. are considered as background. The subclass of the monoclonal antibodies was determined in an enzyme-linked immunosorbent assay. Ascitic fluids at a dilution of 1:10,000 were exposed to plates coated with the 56-residue fragment. (Rabbit antisera against the subclass determinants and a goat anti-rabbit (Ig) horseradish peroxidase-labelled immunoglobulin preparation were from Nordic Immunological Laboratories.) 5-Aminosalicylic acid was used as substrate.

antibodies¹⁴ prepared against the 56-residue fragment recognize antigenic sites on the corresponding segments of intact interferon molecules.

Three BALB/c mice were immunized against the 56-residue fragment, their spleen cells pooled and fused to cells of the mouse myeloma line Fo (ref. 15), and hybrid cells selected for production of fragment-binding antibodies as described in Table 1 legend. Twenty-eight hybrid cell cultures obtained from the fusion of 1.5×10^8 spleen cells with 0.75×10^8 myeloma cells were found to produce proteins that bound to the fragment. Sixteen of the originally positive cultures were cloned twice; of these clones, five produced proteins that cross-reacted with the unrelated antigen ovalbumin, the remaining 11 synthesizing specific antibodies of subclasses IgG1, IgG2a and IgG3, all containing κ light chains.

Further analysis of the binding properties of these specific antibodies in a radioimmunoassay using the synthetic carboxyl-terminal 96- and 33-residue fragment and bacterially produced IFN- α_1 and - α_2 (Table 1) suggested that the hybridoma antibodies fell into three classes: antibodies of clone III/21 bound to all fragments and both interferons; antibodies of clones I/7, II/3, II/11, II/14, VII/8, XXI/5 and XXIV/11 bound to various degrees to all fragments but not to the interferons; the products of clones I/5, II/20 and III/17 bound exclusively to the 56-residue fragment. Thus, at least three different epitopes are recognized on the 56-residue fragment, one of which is accessible to a distinct monoclonal antibody also on natural interferon.

Ascitic fluid from mice bearing tumours of clone III/21 was tested for neutralization of the antiviral effect of IFN- α_1 and - α_2 on three different target cells (human larynx carcinoma line CCL 23, bovine kidney cells and mouse L-cells) on which both interferons show cross-reactivity¹⁶, using Mengo virus, vesicular stomatitis virus or encephalomyocarditis virus as challenge. No reduction of the interferon titres was found after preincubation with ascitic fluid from clone III/21 diluted 1:20, whereas at a dilution of 1:10 the ascitic fluid neutralized a maximum of 3 IFN units and at 1:5 it neutralized a maximum of 9 IFN units. This minor reduction of the interferon titres, however, may have been partly caused by ascitic fluid components other than the monoclonal antibody. The fact that the antibodies raised against the synthetic 56-residue fragment essentially did not neutralize the interferon activity was compatible with the observation that

none of the synthetic fragments inhibited viral growth in the cell lines mentioned above.

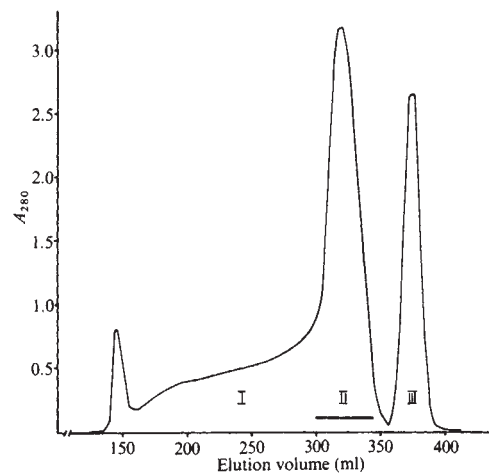
Human leukocyte interferon extracted from bacteria is largely contaminated by prokaryotic proteins. As mouse hybridomas secreting monoclonal antibodies to interferon represent a rich source of material for the preparation of affinity columns, we investigated the efficacy of affinity chromatography for the purification of human interferon produced in bacteria.

Mice were injected with cells of clone III/21 and their ascitic fluids, binding to IFN- α_2 -coated plates with a titre of 10^{-5} , were pooled. The IgG fraction was isolated and coupled to CNBr-activated Sepharose 4B. A column with a capacity of 2×10^7 reference units of IFN- α per ml of gel was loaded with partially purified IFN- α_2 (*E. coli*) (Fig. 3, slot 2) which had a specific activity of 3×10^6 units per mg of protein. The column was eluted with phosphate-buffered saline (PBS, pH 7.2), followed by McIlvaine's buffer (pH 2.6). At neutral pH most of the interferon was retained. The material eluting at pH 2.6 was enriched in interferon (4.8×10^7 units per mg of protein) and low molecular weight (M_r) proteins and was almost free of high molecular weight proteins as shown by SDS-polyacrylamide gel electrophoresis (Fig. 3, slot 3). Re-chromatography of this material in identical conditions gave purified interferon with a specific activity of 8.8×10^7 units per mg of protein. Gel electrophoresis of the fraction eluting at pH 2.6 showed a very strong interferon band corresponding to a M_r of 19,400, a minor band at M_r 17,800, and a very weak band at M_r 13,000 (Fig. 3, slot 4).

The synthetic carboxyl-terminal 56-residue fragment of human IFN- α_1 was not homogeneous: although its amino acid composition as well as chromatographic and electrophoretic purity were satisfactory (the good recovery of tryptophan is particularly noticeable), minor deviations from the target sequence caused mainly by deletions at various stages of the synthesis could not be excluded. Further purification of the 56-residue fragment will involve affinity chromatography on an antibody column and HPLC. However, the CD measurements indicated that the synthetic IFN 111-166 had a considerable amount of stable structure.

Synthetic fragments of natural proteins may be suitable for raising antisera that cross-react with the parent molecules^{17,18}. The present study describes the first production of a monoclonal antibody against a synthetic polypeptide, the linear carboxyl-

Fig. 2 Fractionation of the carboxamidomethylated carboxyl-terminal 56-residue fragment of human IFN- α_1 (105 mg) on a Sephadex G-50 column (97.5 $\times$ 2.4 cm) in 0.05 M CH₃COOH. I, disulphide dimer and aggregates; II, monomeric carboxamidomethylated 56-residue fragment (the bar indicates the elution range of the fraction that was re-chromatographed); III, excess iodoacetamide. The carboxamidomethylated 56-residue fragment was obtained as follows⁷⁻⁹. Tertiary butyloxycarbonyl (Boc) amino acids were coupled in a threefold molar excess based on the amount of carboxyl terminal glutamic acid esterified to the resin. Each coupling reaction was repeated with a twofold molar excess of the Boc amino acid. The side chains of methionine and tryptophan were unprotected. To prevent their alkylation or oxidative cleavage during the acid deprotection step, 10% anisole and 2% β -mercaptoethanol were present in the deblocking mixture (40% trifluoroacetic acid in CH₂Cl₂) after addition to the peptide resin of Boc-Met and Boc-Trp, respectively. At the end of the synthesis the yield of protected 56-residue peptide resin was 85.8%. The synthetic polypeptide was deprotected and cleaved from the resin by anhydrous HF in the presence of anisole (sixfold molar excess per protecting group) and free tryptophan (twofold molar excess per tryptophan residue) at 0 °C for 30 min and then extracted by 1 M CH₃COOH. After lyophilization it was dissolved in 0.05 M NH₄HCO₃, 8 M urea and dialysed against 0.05 M NH₄HCO₃. Decreasing urea concentration caused the synthetic 56-residue polypeptide to precipitate, and lyophilization gave the crude interferon fragment. The yield of the cleavage step was 82.9%. The synthetic fragment was treated with a 100-fold molar excess of β -mercaptoethanol in 5 M urea at pH 4 overnight and the reduced protein was isolated by gel filtration on Bio-Gel P-2 in 0.1 M CH₃COOH. To the pooled protein fractions 0.05 M NH₄HCO₃ was added to give a final protein concentration of 1 mg ml⁻¹. The sulphhydryl group of Cys 139 was blocked by reaction with a 10-fold molar excess of iodoacetamide at pH 7 and the product was fractionated on Sephadex G-50 as shown above. After re-chromatography the carboxamidomethylated 56-residue fragment was obtained in 52.5% yield based on the amount applied to the first column. It was purified further by adsorption chromatography on silica gel 60 using *n*-butanol, acetic acid, pyridine and water (15:3:10:12) as eluant.



terminal 56-residue fragment of human IFN- α_1 . The cross-reactivity of antibodies of clone III/21 with the synthetic interferon fragments 134–166 and 71–166 and with complete human IFN- α_1 and - α_2 (derived from *E. coli*) suggested that there was a common antigenic site of very similar fold that was (largely) located between residues 134 and 166 of the interferon sequence, and accessible on the natural interferon molecules. Monoclonal antibodies that bound the synthetic fragments only, probably recognized structures that were either absent or not accessible on natural interferons.

A small library of monoclonal antibodies against leukocyte interferons already exists. A monoclonal antibody described by Secher and Burke¹⁹ was raised against human leukocyte interferon and selected for its capacity to neutralize the antiviral effect of this protein. It did not cross-react with human fibroblast or mouse interferon²⁰. As the antibodies prepared to the synthetic carboxyl terminal 56-residue fragment of IFN- α_1 were essentially non-neutralizing, the binding site of the monoclonal antibody of Secher and Burke was either located between residues 1 and 110 or was constituted by amino acid residues scattered over a large part of the interferon sequence including

the region from 111 to 166. It was also possible that a neutralizing site at the carboxyl terminus of human leukocyte interferon was not present on the synthetic fragment because of conformational differences. Another monoclonal IgG specific for human leukocyte interferon was obtained by Montagnier *et al.*²¹, while Staehelin and co-workers²² prepared 13 monoclonal antibodies to human leukocyte interferon which gave distinct binding patterns with purified interferon species and of which three were non-neutralizing. The monoclonal antibody described here bound (at least) human IFN- α_1 and - α_2 derived from *E. coli* and was used to prepare an affinity column from which purified human IFN- α_2 (*E. coli*) with a specific activity of $\sim 10^8$ units per mg of protein was isolated. Ultimately, a whole library of monoclonal antibodies may be available to map the antibody and receptor binding sites of the various interferons.

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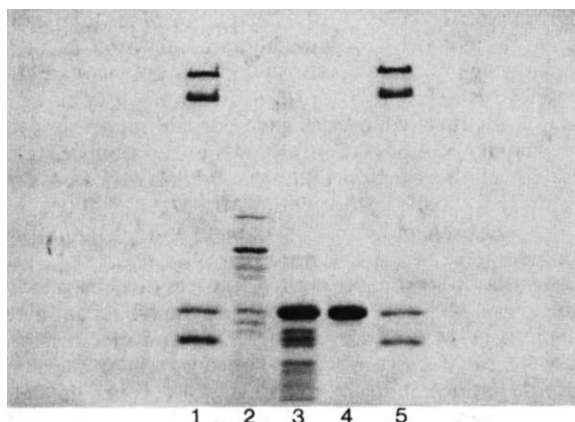


Fig. 3 Gel electrophoretic analysis of *E. coli*-derived human IFN- α_2 before and after affinity chromatography on a column containing a monoclonal antibody prepared against the carboxyl terminal 56-residue fragment of human IFN- α_1 . Gel, 0.1% SDS-17% polyacrylamide; 25 μ g of protein were applied per slot. Staining was with Coomassie brilliant blue. 1, 5, Protein markers (from top to bottom: bovine serum albumin, catalase, β -lactoglobulin, cytochrome *c*). 2, Partially purified interferon applied to the antibody column. 3, Material eluted with McIlvaine's buffer, pH 2.6; after neutralization it was re-chromatographed on the antibody column. 4, Re-chromatographed material eluting at pH 2.6; the strong band at molecular weight 19,400 is interferon.

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Carrier-dependent and carrier-independent transport of anti-cancer alkylating agents

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While it is understandable that proliferating cells should be more sensitive than resting cells to antimetabolites—drugs that derange DNA synthesis—it is not so obvious that other anti-cancer drugs, in particular the alkylating agents, should act in the same way¹⁻³. The greater sensitivity of proliferating cells to such agents is not confined to malignant tissues: normal haematopoietic cells show increased susceptibility after recruitment into cell division⁴. Moreover, the various alkylating agents have different effects on proliferating nonmalignant cells; in normal bone marrow, recovery is more rapid⁵ and greater⁶ after treatment with some drugs (for example, cyclophosphamide and nitrogen mustard) than others (such as nitrosoureas and mitomycin C). We now provide evidence in support of the hypothesis that there are two distinct groups of alkylating agent: those whose uptake into the cell depends on a membrane transport mechanism (that is, carrier-dependent) and those whose uptake is not so dependent (carrier-independent).

An assay which uses the mitogen phytohaemagglutinin (PHA) to induce proliferation in tissue culture of human peripheral T lymphocytes⁷⁻⁹ was developed for these experiments¹⁰. It can be seen (Fig. 1) that ³H-thymidine uptake was very low before PHA stimulation but increased steadily (as measured by continuous label incorporation) in the 18 h after its addition. Therefore, in this assay, unstimulated human peripheral T lymphocytes are considered a 'resting' cell population and after 18 h of exposure to PHA they are considered to be actively proliferating. To test the effects of alkylating agents on these two populations of cells, various drug concentrations were added either 60 min before or 18 h after PHA stimulation. An initial experiment used melphalan (L-phenylalanine mustard), which has recently been shown to be actively taken up by an amino acid transport system in both rodent cells and human lymphocytes¹¹⁻¹³. Figure 1 shows the effect of a 60-min exposure to melphalan at various periods (between 0 and 26 h) after PHA stimulation. Initially, melphalan caused only ~30% death of resting human peripheral T lymphocytes, but its toxicity steadily increased as PHA-induced proliferation increased, resulting in a maximum of ~90% death 8 h after addition of PHA.

We then determined quantitatively the cytotoxicity of various alkylating agents and X rays, using a series of increasing drug concentrations to obtain reasonably complete (2-3 log death) survival curves. In each case the cells were exposed to the agent for 60 min (an arbitrary exposure time) either at time 0 (resting cells just before PHA exposure, solid circles in Figs 2 and 3) or 18 h after PHA-induced proliferation (cycling cells, open circles in Figs 2 and 3). We obtained two clearly distinguished survival patterns. The first of these we term carrier-dependent (see Fig. 2). Carrier-dependent toxicity is characterized by dose-dependent cell death but with a substantially greater shoulder on the survival curve of resting cells. The shoulder is reduced or eliminated when the cells are exposed to the carrier-dependent agent after induction of cell cycling. Melphalan, nitrogen mustard and *cis*-dichlorodiammineplatinum (CDDP) all gave this pattern. These three agents are highly water-soluble and two are known to be transported into cells by membrane carriers (melphalan by amino acid carriers¹¹⁻¹³ and nitrogen mustard by a choline carrier^{14,15}). We have also found that amino acids can protect cells against CDDP, suggesting that the latter is probably also taken up by an amino acid transport mechanism¹⁶, which may explain its nephrotoxicity¹⁷. A fourth water-soluble alkyl-

ating agent, phosphoramide mustard (the active form of cyclophosphamide), proved almost inert (at concentrations up to 100 µg ml⁻¹) against both resting and cycling human T cells, a feature which may explain its well-documented differential sparing of T cells *in vivo*¹⁸.

The second survival pattern, which we term carrier-independent toxicity, is shown in Fig. 3. All the carrier-independent

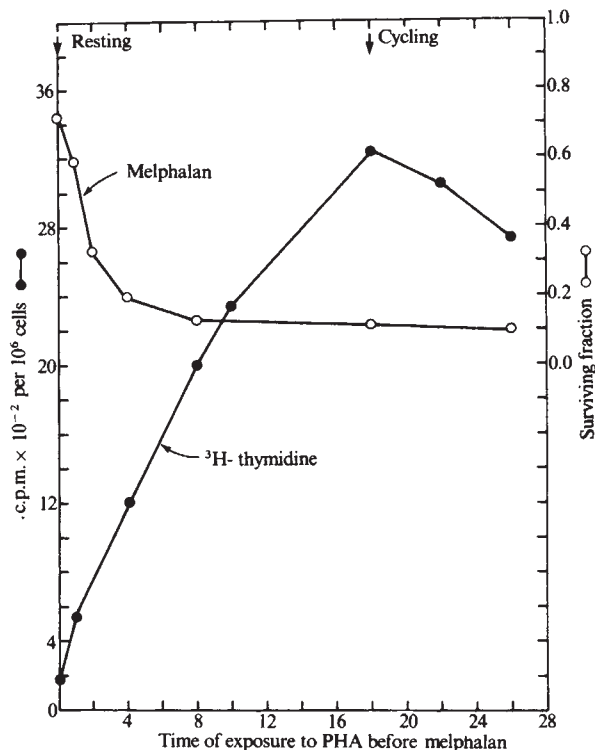
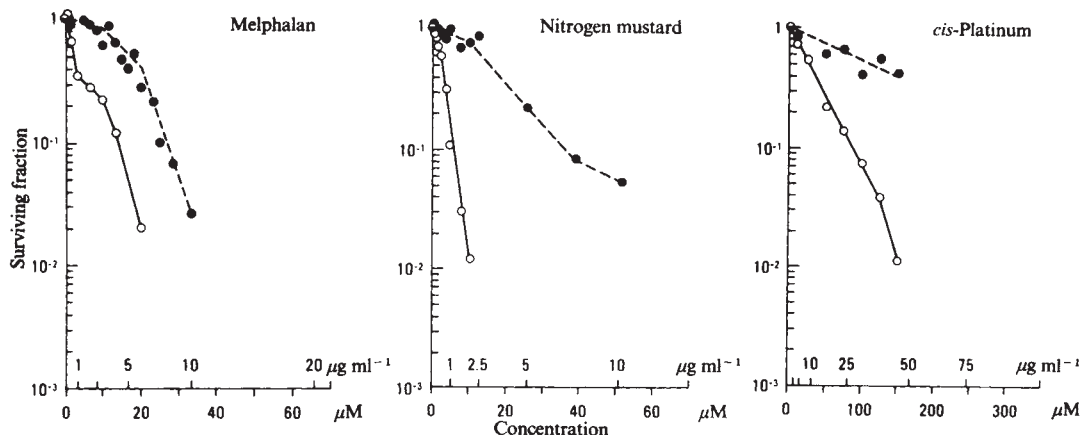


Fig. 1 Effect of PHA exposure time on the survival of normal human peripheral T lymphocytes exposed to melphalan (4 µg ml⁻¹) for 1 h (○) and on the incorporation of ³H-thymidine continuous label (●). The assay procedure for determining drug cytotoxicity of normal human T lymphocytes was as follows. Peripheral blood from a healthy adult was collected with 50 U ml⁻¹ heparin and the mononuclear cells were isolated by Ficoll-Paque (Pharmacia) gradient centrifugation. Cells from the interphase containing >95% lymphocytes were washed three times in Hank's balanced salt solution (HBSS; Gibco) and resuspended at 10⁶ cells ml⁻¹ in McCoy's 5a medium containing 15% heat-inactivated fetal bovine serum (FBS; Gibco). Aliquots (1 ml) of the cell suspension were seeded into 16 × 125 mm tubes (Falcon 3033); replicate tubes were used for determining cell survival or DNA synthesis. PHA (HA15 Wellcome) was freshly prepared at 2% in complete medium and 1 ml added to each tube at time 0; one set of tubes (●) contained [³H]thymidine (43 Ci mmol⁻¹, 10 µCi ml⁻¹; Amersham). At the times indicated, one tube from each set was removed and DNA synthesis determined (●). The other tube (○) was exposed to melphalan (NSC 8806, freshly prepared at 4 µg ml⁻¹ final concentration in complete medium) for 1 h, followed by washes and re-exposure to PHA for the remaining time necessary to complete an 18-h PHA exposure for colony formation. The cells were then washed in HBSS and syringe-resuspended in 2 ml complete medium to disaggregate the cells. To each tube were added 1 ml of 1% PHA in complete medium and 2 ml 2.6% methylcellulose in complete medium. Cells were plated in 35-mm dishes (Falcon) to give a final concentration per plate of 5 × 10⁵ cells in 0.2% PHA, 1.0% methylcellulose. PHA-induced colonies containing >50 cells were counted with an inverted microscope at day 7. Plating efficiencies for non-treated controls in both the resting and cycling sets were consistently ~0.1%. Surviving fractions were determined by assigning a value of 1.0 to the untreated controls. DNA synthesis was determined by the method of Evans and Norman⁵². Briefly, the labelled cells were washed three times in cold PBS containing 100 µg ml⁻¹ thymidine (Sigma) and resuspended in 10 ml cold PBS. Cell number was determined and cells placed on Millipore GS 0.22-µm filters. Filters were washed with 5% trichloroacetic acid, then 95% ethanol, and air-dried overnight and placed in vials with 10 ml scintillation fluid.

Fig. 2 Effect of carrier-dependent agents on the survival of normal human lymphocytes before (●) and after PHA stimulation for 18 h (○). The overall assay procedure is outlined in Fig. 1 legend. The two different time points for the 60-min exposure are indicated by arrows in Fig. 1. All drugs were freshly prepared before use. Melphalan (NSC 8806) was dissolved at 10 mg ml⁻¹ in ethanol/HCl (9:1). Nitrogen mustard (Mustargen) was obtained as a 1 mg ml⁻¹ solution in PBS; *cis*-platinum (Platinol; Bristol) was obtained as 10 mg lyophilized sample and reconstituted with sterile double distilled water. All dilutions were made in complete media.



drugs gave identical survival curves for resting and cycling cells and all are highly lipid-soluble. Those drugs which showed this pattern (Fig. 3) include the nitrosoureas 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and methyl-CCNU [1-(2-chloroethyl)-3-trans-(4-methyl-cyclohexyl)-1-nitrosourea] (plus CCNU, data not shown), procarbazine, busulfan (data not shown), mitomycin C, an amphipathic agent freely soluble in both water and lipids¹⁹, and ACNU [1-(4-amino-2-methyl-pyrimidin-5-yl)-methyl-3-(2-chloroethyl)-3-(nitrosourea)], an amphipathic nitrosourea with delayed marrow toxicity²⁰. These results

suggest that the extent of lipid solubility (which would override a carrier mechanism) may dictate the type of cell survival pattern. X rays (whose entry into cells is independent of any 'carrier') also showed a carrier-independent pattern. As these nitrosoureas^{21,22} and mitomycin C²³ are bifunctional alkylating agents and cross-link DNA in a manner similar to that of the classical mustards, it seems unlikely that it is the nature of the molecular damage which results in the different survival patterns. Repair of DNA damage in resting cells (that is, before cycling) also cannot explain the relative resistance of resting cells

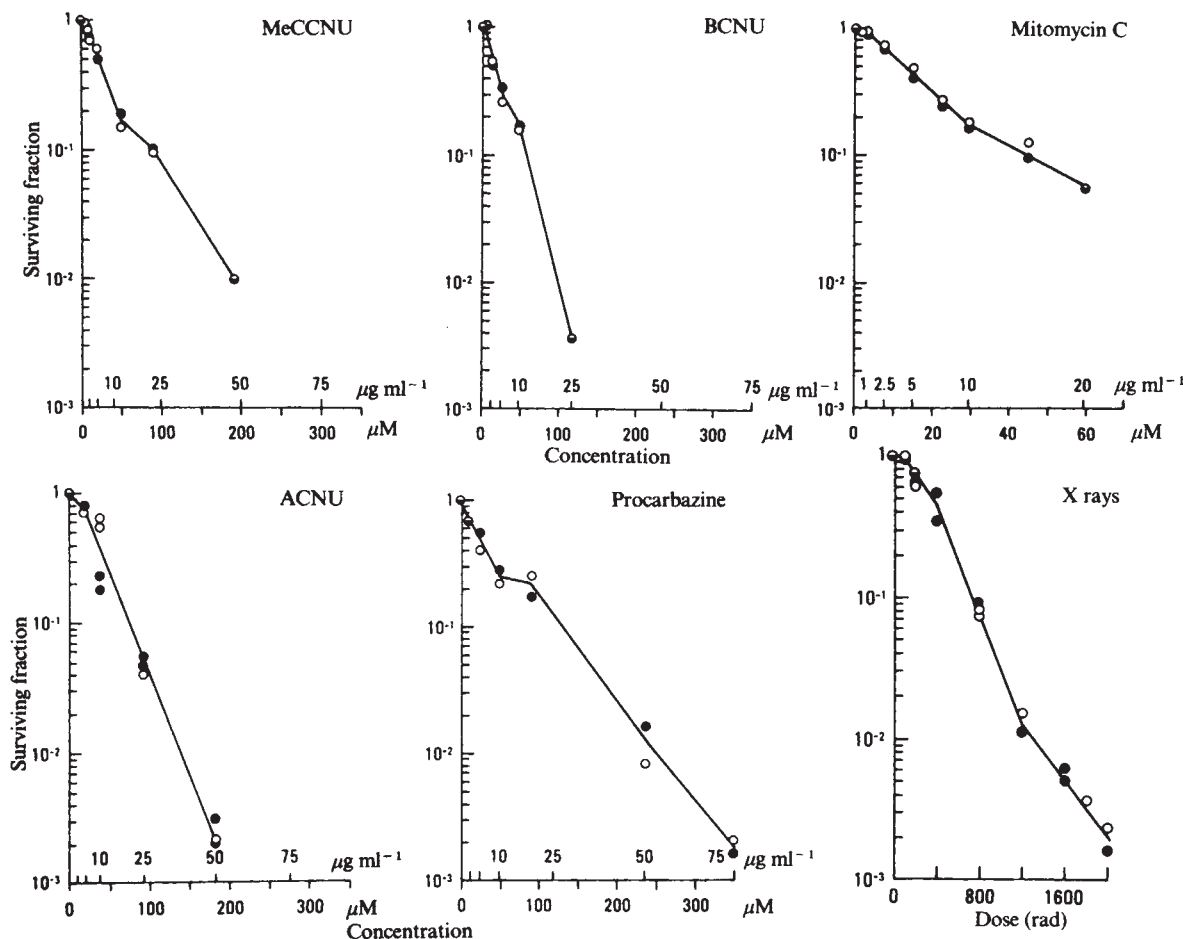


Fig. 3 Effect of carrier-independent agents on the survival of normal human lymphocytes before (●) and after PHA stimulation for 18 h (○). The assay procedure is outlined in Fig. 1 legend. All drugs were freshly prepared before use. Methyl-CCNU (MeCCNU; NSC 95441), BCNU (NSC 409962), ACNU (NSC D245382) and procarbazine (NSC 77213) were dissolved at 1 mg ml⁻¹ in absolute ethanol. Mitomycin C (Mutamycin; Bristol) was obtained as 5 mg in sterile distilled water. All dilutions were made in complete media. X-ray exposure was at room temperature in air on a Picker orthovoltage 280 keV machine, using an HVL 1.0 mm copper filter with an output of 150 rad min⁻¹.

to carrier-dependent drugs, as the carrier-independent alkylating agents would be expected to show the same evidence of cellular repair. The feature which dictates the shape of the survival curve and governs the (relative) independence/dependence of toxicity on the cell's proliferative state seems to be the drug's ability to penetrate the cell by simple diffusion into cellular lipids and lipoproteins^{24,25}.

We believe these two patterns are reflected in the two recovery patterns shown by bone marrow after exposure to alkylating agents. When delivered as a bolus, the recovery of marrow from carrier-dependent agents is usually fairly rapid such that cyclical therapy can be repeated about every 21 days^{5,26-28}. The equivalent administration of a lipid-soluble nitrosourea^{20,29}, mitomycin C^{30,31}, or X rays²⁸ is followed by a considerably prolonged marrow recovery period of 4-6 weeks. In the rat this delayed recovery pattern has recently been shown (for CCNU) to relate to marrow stem-cell killing independent of the proliferative state of the pluripotential marrow stem cells³². Clinically, all the carrier-independent agents (those cited in Fig. 3 legend, plus CCNU and busulfan) are associated with delayed marrow recovery and also with cumulative marrow damage, that is, a steady irreversible depletion of marrow reserve with progressive dosage^{5,20,33-35}. On the other hand, the carrier-dependent agents produce relatively less marrow damage and show much more rapid marrow recovery^{6,28}. The only apparent exception to this rule is melphalan^{31,36}; we believe this discrepancy is due to the prolonged administration schedule that has been used for melphalan in man.

This binary model may also be relevant to the means by which tumour cells develop resistance. It predicts that a significant source of resistance to carrier-dependent alkylating agents could stem from somatic mutations that yield cells whose membrane carriers are either fewer in number or have reduced drug affinity. A contribution to resistance by reduced transmembrane transport has recently been shown for melphalan¹³. As CDDP seems to be transported by a similar pathway¹⁶, its partial cross-resistance with melphalan^{37,38} is perhaps expected.

For carrier-independent agents, cell uptake seems to be independent of membrane events and the existence of somatic mutants conferring significant resistance at the membrane level is unlikely. This suggests that 'resistance' to carrier-independent alkylating agents can stem only from intracellular sources, for example, by reduced intracellular activation of the agent (if such is needed), selection of cells with greater DNA repair mechanisms³⁸⁻⁴² or by nonspecific protective effects such as the selection of mutants with elevated thiol levels⁴³. The reduced tendency of carrier-independent drugs to induce resistance⁴⁴, and their high activity in some rodent models⁴⁵, make carrier-independent agents superficially attractive. However, the data in Fig. 3 indicate that such drugs do not have any true differential cytotoxicity between resting and cycling normal stem cells. These properties reduce their overall clinical potential considerably as regimens exploiting the considerable recruitment potential of resting normal tissues are precluded.

These data suggest that the proliferation dependency of alkylating agent cytotoxicity stems from two unequal and disparate sources: (1) a major dependence, limited to carrier-dependent drugs, on enhanced transport of the drug into the cell because it is, in part, a stereochemical analogue of a normal compound (for example, amino acid, choline) used in cell biosynthesis; (2) a minor dependence (shared by both carrier-dependent and carrier-independent drugs) attributable to inherent sensitivity of replicating DNA to alkylating agents. We recently showed⁴⁶ that the cytotoxicity exerted by alkylating agents seems to be a function of the number of DNA replicons that encounter unrepaired alkylated sites on the DNA. These data, together with recent studies⁴⁷ on the mechanism of recovery from CDDP damage of resting tissue culture cells suggest this to be the molecular basis for the enhanced sensitivity of cycling cells to both carrier-dependent and carrier-independent alkylating agents.

There are several implications of this model: (1) carrier-dependent agents are likely to be more useful clinically than those that are carrier-independent, and new, useful carrier-dependent alkylating agents can probably be synthesized that will exploit membrane carriers not available to present agents (for example, bases, sugars, other amino acids, polyamines); (2) the minimal dose of each carrier-dependent agent used in each treatment cycle should probably be at or just beyond the point where the differential effect of the agent against resting versus cycling cells is first maximized; (3) treatment schedules which include and serially alternate several carrier-dependent agents known to be lacking in cross-resistance are predicted to be most useful, as this would minimize the outgrowth of drug-resistant (membrane) mutants^{48,49}; (4) the inclusion of carrier-independent agents in these schedules is likely to be harmful, except in the rare event where a specific advantage such as central nervous system penetration or hypoxic activation⁵⁰ exists, or where the net tumour volume is minimal and ('adjuvant') non-selective chemotherapy might be effective; (5) therapies designed to enhance proliferation-dependent carrier-dependent drug killing (for example, combinations of carrier-dependent alkylating agents and clinically tolerable methylated xanthines, for example see ref. 51) might also usefully be examined.

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Streptozotocin and alloxan induce DNA strand breaks and poly(ADP-ribose) synthetase in pancreatic islets

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Streptozotocin, which produces diabetes mellitus in experimental animals¹⁻³, has been reported to reduce the level of NAD in pancreatic islets^{4,5} and to inhibit islet synthesis of proinsulin⁶. The decrease in NAD is due to increased NAD degradation mediated by islet nuclear poly(ADP-ribose) synthetase^{7,8}. Evidence suggests that poly(ADP-ribose) synthetase is activated when DNA is fragmented⁹⁻¹⁷. Here we describe that both streptozotocin and alloxan, which also produces experimental diabetes mellitus^{1,2}, cause DNA strand breaks which stimulate nuclear poly(ADP-ribose) synthetase, thereby depleting intracellular NAD and inhibiting proinsulin synthesis in isolated pancreatic islets of rats.

Islets isolated from rat pancreas were incubated with streptozotocin or alloxan for 5–20 min in Krebs-Ringer's bicarbonate medium and velocity sedimentation of DNA from the islet cells was carried out in an alkaline sucrose gradient. DNA from islets incubated for 10 min without the diabetogenic agents was recovered as a single peak near the bottom of the gradient, the position at which undamaged DNA sediments (Fig. 1a, e). However, after only 5 min incubation with 2 mM streptozotocin or 1 mM alloxan, a considerable amount of DNA sedimented as a broad peak in the middle of the gradient with a concomitant decrease in undamaged DNA (Fig. 1b, f); after 10–20 min incubation, the DNA was almost completely fragmented (Fig. 1c, d, g, h). The effect of the two agents on islet DNA fragmentation was dose dependent. These results clearly indicate that streptozotocin and alloxan produce strand breaks in islet DNA.

DNA fragmentation in mammalian cells has been reported to activate nuclear poly(ADP-ribose) synthetase⁹⁻¹⁵, and there is direct evidence^{16,17} that poly(ADP-ribose) synthetase purified from bovine thymus is activated only when the enzyme is bound to fragmented or nicked DNA. Therefore, we prepared a nuclear fraction from islets incubated in conditions causing breaks in islet DNA, and assayed poly(ADP-ribose) synthetase activity. As shown in Fig. 2a, b, both streptozotocin (2 mM) and alloxan (1 mM) induced a two- to threefold increase in islet poly(ADP-ribose) synthetase activity, with a peak at 10 min. Streptozotocin has also been shown to cause an approximately 1.2–3-fold increase in nuclear enzyme activity of *Physarum polycephalum*¹⁸ and HeLa cells¹⁹; and as much as a 10-fold increase in enzyme activity has been observed in γ -irradiated permeable cells²⁰. The procedure used to isolate the nuclei is reported^{10,20} to cause DNA strand breaks and to activate poly(ADP-ribose) synthetase. We therefore examined a DNA sedimentation profile of the islet nuclear fraction. We found that the isolation procedure *per se* caused some DNA breaks, but that nuclear DNA from islets incubated with alloxan or streptozotocin was fragmented to a greater degree than that induced by the nuclear isolation procedure alone (data not shown). This indicates that the low, two- to threefold increase in islet nuclear poly(ADP-ribose) synthetase activity was probably due to the high background of DNA breaks resulting from the isolation procedure.

Cellular NAD content was reduced by either 2 mM streptozotocin or 1 mM alloxan within 20 min of incubation (Fig. 2c, d), and remained almost unaltered for 60 min. There was striking temporal correlation between the decrease in level of islet NAD and the increase in islet poly(ADP-ribose) synthetase activity. These results indicate that both alloxan and streptozotocin cause DNA strand breaks which result in an increase in poly(ADP-ribose) synthetase activity, thereby depleting islet NAD level. However, it has been suggested that alloxan may not be toxic to islets because it decreases the NAD level^{2,3}.

Next, we examined the effect of inhibitors of poly(ADP-ribose) synthetase on streptozotocin- or alloxan-reduced islet NAD content. The inhibitors used were nicotinamide and picolinamide, which are potent inhibitors of islet nuclear poly(ADP-ribose) synthetase⁷. The results are shown in Table 1. Alloxan (1 mM) decreased islet NAD level to 18% of the control level (without alloxan). Nicotinamide (2 mM) or picolinamide (2 mM) completely abolished the alloxan-induced decrease in islet NAD level. As previously described^{5,7}, the same was true for the decrease in islet NAD level induced by streptozotocin. Addition of nicotinamide or picolinamide alone produced only a slight increase in islet NAD level.

The ability of islets to synthesize proinsulin is considered a crucial marker for evaluation of diabetogenicity of alloxan and streptozotocin^{21,22}. As shown in Table 2, 1 mM alloxan reduced proinsulin synthesis to 9% of the control level; nicotinamide (2 mM) or picolinamide (2 mM) reversed this decrease to 35% or 27% of the control value. The decrease in proinsulin synthesis induced by 0.5 mM alloxan (30% of the control) was almost completely abolished by nicotinamide or picolinamide. The decrease in proinsulin synthesis induced by streptozotocin (1 or 2 mM) was also significantly reversed by the addition of either nicotinamide or picolinamide, as described elsewhere⁷. In addition to these *in vitro* experiments with isolated rat islets, *in vivo* experiments on rats confirmed that nicotinamide and picolinamide prevent development of alloxan- as well as streptozotocin-induced diabetes (unpublished data).

This study demonstrates that streptozotocin and alloxan cause DNA strand breaks to stimulate nuclear poly(ADP-ribose) synthetase, thereby depleting intracellular NAD and inhibiting proinsulin synthesis. Inhibitors of islet nuclear poly(ADP-ribose) synthetase such as nicotinamide and picolinamide were found to reverse the reduction in NAD level and also the inhibition of proinsulin synthesis. These inhibitors may prevent the diabetogenic action of alloxan and streptozotocin by inhibiting NAD degradation through poly(ADP-ribose). Other species of poly(ADP-ribose) synthetase inhibitors, such as 3-aminobenzamide, 3-nitrobenzamide and 3-methoxybenzamide, were found to protect against the depression of proinsulin synthesis induced by streptozotocin or alloxan (H.Y., A. Kawamura and H.O., in preparation). Methylxanthines may protect against the action of alloxan on islets²³ in a similar way, because they also have been reported to inhibit poly(ADP-ribose) synthetase^{24,25}.

Thiols²⁶ and radical scavengers²⁷ can also protect against the diabetogenic action of alloxan. It has been suggested that hydroxyl radicals generated from alloxan mediate its diabetogenic action²⁷⁻²⁹. More recently, we observed that strand breaks in islet DNA by alloxan were abolished in the presence of superoxide dismutase (Y.U., H.Y. and H.O., in preparation). Thus it is likely that the free radicals generated from alloxan damage islet cell DNA, resulting in activation of poly(ADP-ribose) synthetase.

The increase in islet poly(ADP-ribose) synthetase activity induced by streptozotocin or alloxan resulted in a significant depletion of islet NAD content. This reduction in intracellular NAD to such a non-physiological level may severely affect islet cell functions, including proinsulin synthesis. In addition, certain viruses or physical factors such as γ rays, which are known to cause DNA strand breaks and to stimulate poly(ADP-ribose) synthetase^{11-15,30,31}, may be expected to be aetiological agents of insulin-dependent diabetes⁸.

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Table 1 NAD content of islets incubated in the presence of alloxan or streptozotocin with or without the addition of nicotinamide or picolinamide

Addition	Total NAD in PCA extract (pmol)	^{14}C -NAD in PCA extract (c.p.m. $\times 10^4$)	% Recovery	Islet NAD content (pmol per islet)
None	351	4.16	93.2	2.84 (100)
Alloxan (1 mM)	141	4.37	97.9	0.52 (18)
Alloxan (1 mM) and nicotinamide (2 mM)	369	4.26	95.5	2.94 (104)
Alloxan (1 mM) and picolinamide (2 mM)	364	4.35	97.6	2.81 (99)
Streptozotocin (2 mM)	116	4.10	92.0	0.34 (12)
Streptozotocin (2 mM) and nicotinamide (2 mM)	365	4.25	95.4	2.91 (102)
Streptozotocin (2 mM) and picolinamide (2 mM)	380	4.40	98.6	2.93 (103)
Nicotinamide (2 mM)	395	4.29	96.1	3.19 (112)
Picolinamide (2 mM)	377	4.19	94.0	3.09 (109)

Batches of 100 islets were incubated at 37 °C for 20 min and NAD content of the islets determined as described in Fig. 2 legend. ^{14}C -NAD (4.46×10^4 c.p.m., 92.1 pmol) was added to each sample before disruption of the islets, and the recovery of ^{14}C -radioactivity in the final preparation used to correct the NAD value for overall recovery. Numbers in parentheses are islet NAD content as a percentage of NAD content in cells without any additions.

Fig. 1 Sedimentation profiles in an alkaline sucrose gradient of DNA from islets incubated with streptozotocin or alloxan. Pancreatic islets of Langerhans were isolated by the method of Okamoto *et al.*³² from male Wistar rats weighing 200–250 g, which were fed *ad libitum*. Batches of 100 islets were incubated for 5–20 min at 37 °C in 100 μl Krebs–Ringer's bicarbonate medium, pH 7.4, supplemented with 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mg ml^{-1} bovine serum albumin (BSA) and 2.8 mM glucose in an atmosphere of 95% O_2 /5% CO_2 (refs 21, 32). Streptozotocin (Upjohn) and alloxan monohydrate (Wako Pure Chemical Industries) were dissolved in distilled water just before addition because of their relatively short half lives^{1,2}. After incubation, islets were suspended in 50 μl of cold 0.9% saline and immediately layered over 0.5 ml lysis solution (1 M NaOH, 0.01 M EDTA, 1% (v/v) Triton X-100) that had just been layered over 14.8 ml of a 5–20% (w/v) linear sucrose gradient containing 0.3 M NaOH, 0.7 M NaCl and 0.01 M EDTA. On the bottom of each gradient was a 1 ml 80% (w/v) sucrose shelf. The loaded gradients were placed in the dark at room temperature for 30 min, then centrifuged at 26,000 r.p.m. at 20 °C for 200 min in a Beckman SW27.1 rotor. After centrifugation, fractions of 33 drops were collected from the gradient. DNA in each fraction was precipitated by adding 2 ml of 20% cold trichloroacetic acid (TCA) with 200 μg BSA as carrier. The precipitate was washed 3 times with cold TCA, once with cold 0.1 M potassium acetate in ethanol, twice with ethanol, then assayed for DNA content by a fluorimetric method³³. Each point represents the percentage of total DNA recovered; recovery was between 85 and 100%. $\blacktriangle$, DNA from islets incubated without streptozotocin for 100 min (a) and with streptozotocin (2 mM) for 5 min (b), 10 min (c) and 20 min (d). $\bullet$, DNA from islets incubated without alloxan for 10 min (e) and with alloxan (1 mM) for 5 min (f), 10 min (g) and 20 min (h). Sedimentation was from left to right. Arrow indicates the position of a bacteriophage λ DNA (3.2×10^7 molecular weight, New England BioLabs).

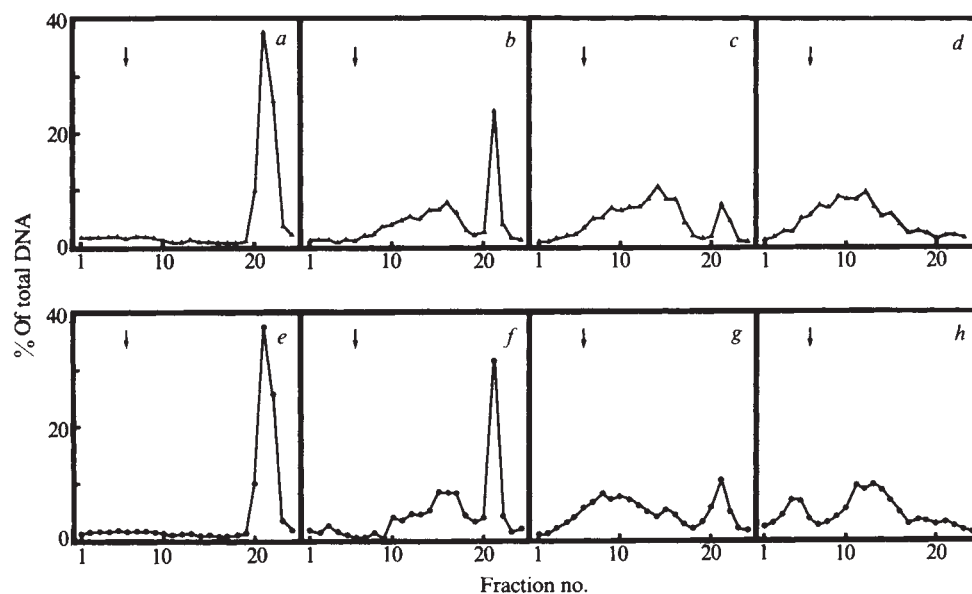


Fig. 2 Effect of streptozotocin or alloxan on poly(ADP-ribose) synthetase activity and NAD content of pancreatic islet cells. Batches of 300 islets were incubated for 0–60 min at 37 °C in conditions described in Fig. 1 legend. After incubation, nuclear fractions were prepared from islets and assayed for poly(ADP-ribose) synthetase activity as described previously⁷. The DNA content of each islet nuclear fraction was determined by a fluorimetric method³³ and used to estimate accurately the enzyme activity. All activities were related to activity at 0 min (10.9 pmol poly(ADP-ribose) synthesized per 10 min per μg islet nuclear DNA). a, Poly(ADP-ribose) synthetase activity in islets incubated with 2 mM streptozotocin (Δ); b, poly(ADP-ribose) synthetase activity in islets incubated with 1 mM alloxan ($\circ$). For determination of islet NAD content, batches of 100 islets were incubated for 0–60 min after which they were disrupted by sonication in 1 ml of cold 0.5 M perchloric acid (PCA). The acid-soluble extract of islets was brought to pH 5.0 with KOH, and NAD content in the extract determined as described elsewhere^{2,7}. The values were corrected for overall recovery by the addition of ^{14}C -NAD (see Table 1 legend), and related to NAD content at 0 min (2.62 pmol NAD per islet). c, NAD content in islets incubated with 2 mM streptozotocin (Δ); d, NAD content in islets incubated with 1 mM alloxan ($\bullet$).

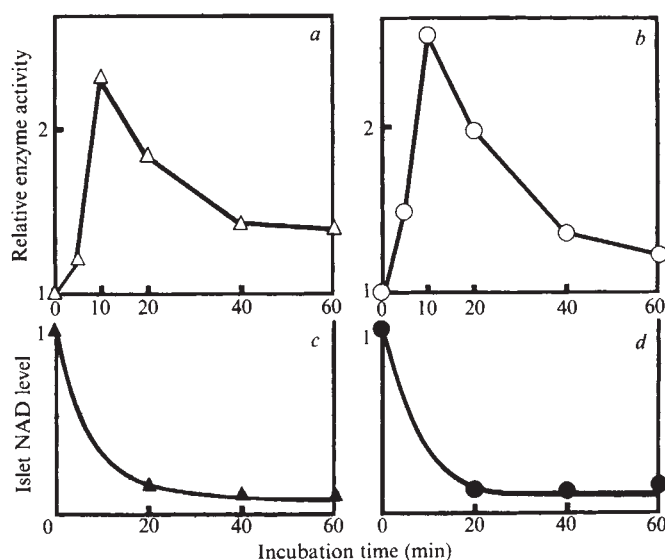


Table 2 Effect of nicotinamide or picolinamide on depression of proinsulin synthesis induced by alloxan or streptozotocin

Addition	³ H-proinsulin synthesized (c.p.m. per h per islet)
None	3,410 (100)
Alloxan (1 mM)	307 (9)
Alloxan (1 mM) and nicotinamide (2 mM)	1,194 (35)
Alloxan (1 mM) and picolinamide (2 mM)	921 (27)
Alloxan (0.5 mM)	1,009 (30)
Alloxan (0.5 mM) and nicotinamide (2 mM)	3,372 (99)
Alloxan (0.5 mM) and picolinamide (2 mM)	2,496 (73)
Streptozotocin (2 mM)	1,187 (35)
Streptozotocin (2 mM) and nicotinamide (2 mM)	2,868 (84)
Streptozotocin (2 mM) and picolinamide (2 mM)	2,568 (75)
Streptozotocin (1 mM)	2,114 (62)
Streptozotocin (1 mM) and nicotinamide (2 mM)	3,096 (91)
Streptozotocin (1 mM) and picolinamide (2 mM)	3,045 (89)
Nicotinamide (2 mM)	3,584 (105)
Picolinamide (2 mM)	3,342 (98)

Batches of 30 islets were preincubated at 37 °C for 5 min in 100 µl of Krebs-Ringer's bicarbonate medium containing 2.8 mM glucose in the presence or absence of the chemicals as indicated, then incubated for 60 min with the addition of 100 µl of medium containing 10 µCi ³H-leucine (specific activity 122 Ci mmol⁻¹; NEN) and 37.2 mM glucose. The final concentration of glucose was 20 mM, at which proinsulin comprises more than one-half the total proteins synthesized *de novo* in islets and gives a major peak on SDS-polyacrylamide gel electrophoresis²². Incubated islets were washed three times with cold Hank's solution, sonicated in 200 µl of 60 mM Tris-phosphoric acid buffer, pH 6.8, containing 1% SDS, 5% 2-mercaptoethanol and 10% glycerol, and heated at 100 °C for 2 min. An 80-µl aliquot of the heated sample was submitted to SDS-polyacrylamide gel electrophoresis. The amount of proinsulin synthesized was calculated by summing the ³H radioactivity incorporated in the proinsulin fractions²². Numbers in parentheses are values for proinsulin synthesis as a percentage of that in islets incubated without any additions.

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Intramolecular flexibility in phenylalanine transfer RNA

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Yeast tRNA^{Phe} is an L-shaped molecule, where each arm is a short segment of double-helical RNA. Examination of the crystal structure has suggested that the molecule may be hinged, perhaps in the region where the anticodon stem meets the D stem¹, or where the two arms of the molecule meet². Because tRNA interacts with a variety of macromolecules during protein synthesis, and because it performs various functions in addition to its role in protein synthesis³, the idea of a hinge is attractive: such conformational flexibility would facilitate functional flexibility. Here we present the results of a theoretical study of the bending of tRNA^{Phe} about the proposed hinge at the junction of the two arms², applying the conformational energy calculation method used previously to examine flexibility in lysozyme⁴ and in DNA⁵. The model is surprisingly flexible, swivelling with two degrees of freedom through angles as large as 30° at an energetic cost of only a few kcal per mol. These energies are comparable with that of a few hydrogen bonds, suggesting that solvent conditions and interactions with other molecules are important in modulating structure and flexibility.

The molecule is modelled on a computer by specifying the coordinates of all the atoms as determined crystallographically, the molecular topology (which atoms are bonded to each other) and a set of empirical energy functions^{6,7} for all the bond lengths, bond angles, dihedral angles, nonbonded contacts (both van der Waals and electrostatic) and hydrogen bonds. Then, treating the two arms as essentially rigid and specifying the location of the hinge or swivel between them, one arm is rotated about that point in increments of 3°; partial structural relaxation is allowed by energy refinement after each bending increment. The resulting sets of coordinates are subjected to several hundred cycles of steepest-descent energy refinement, and the difference between

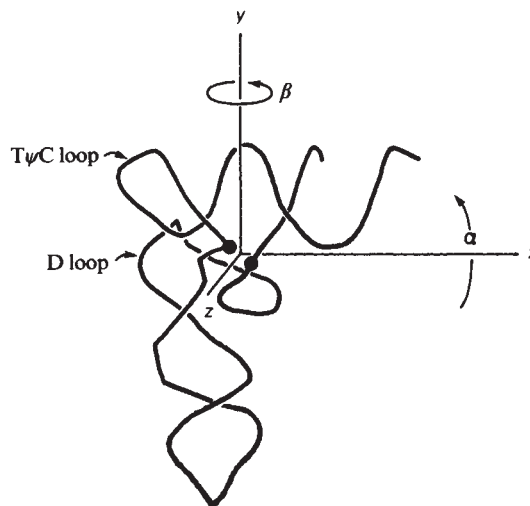


Fig. 1 Backbone of tRNA^{Phe}, showing D and TψC loops, coordinate system centred between phosphates P8 and P49 (●) and the two degrees of freedom. The coordinate system is not that of the crystal structure but has been chosen so that the xy plane is the least-squares best fit plane in the sense that the sum of the squares of the atomic distances from the plane is a minimum. True bending is represented by α, measuring rotations about the z axis, while β designates swiveling, with rotations about the y axis. Positive values of α swing the arms apart and push the D and TψC loops together; positive values of β keep the arms at right angles and pull the D and TψC loops apart.

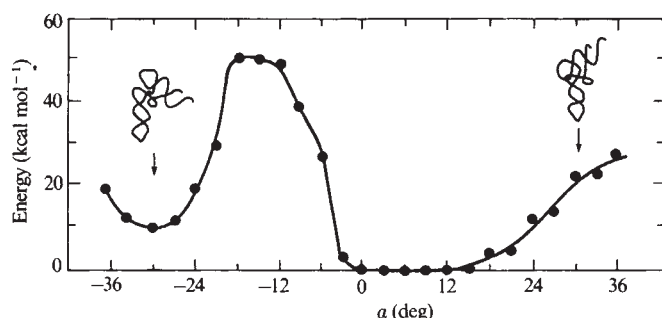


Fig. 2 Dependence of energy on bend angle α . The crystallographic structure defines $\alpha = 0^\circ$ and the backbone conformations are indicated for $\alpha = \pm 30^\circ$. The swing angle is unchanged ($\beta = 0^\circ$) during the bending. In Figs 2 and 3, for clarity, negative energies are shown as $E = 0$; no values below -2 kcal mol^{-1} were observed, and because this is about the limit of resolution and the scatter was random (no clear minima were observed), we do not consider the negative values to be significant.

the energies of the bent and unbent models is assumed to be the energy cost of bending.

For this study, we used the $2.5 \text{ \AA}$ crystallographic structure of Jack *et al.*^{7,8} obtained from the Brookhaven Protein Data Bank⁹. The empirical energy parameters are essentially those of Levitt^{5,10}, as subsequently modified for tRNA⁷, but partial electrostatic charges¹¹ and approximate corrections for solvent dielectric effects¹² have been included. Neither Mg^{2+} nor any other cation has been included, as the study was intended to assess the intrinsic molecular flexibility; we hope to examine the effects of polyvalent cations in future work. There are 79 hydrogen bonds in the model, including the 56 interbase bonds of the cloverleaf secondary structure, 12 bonds between $\text{O}2'$ of one ribose and an oxygen atom ($\text{O}4'$ or $\text{O}5'$) of the successive ribose, and 11 tertiary bonds. This represents all the commonly accepted hydrogen bonds¹³ except for 10: 7 bonds between the D and T ψ C loops (2 between G18 and $\psi 55$; 3 between G19 and C56; 1 between G57 and R18; 1 between G57 and R19) and 3 within the T ψ C loop (2 between T54 and A58; 1 between G57 and R55). The interloop bonds are proposed to serve as a catch mechanism which opposes bending, while the bonds within the T ψ C loop were found to become highly distorted as the loop folds to accommodate the bending motions. As these 10 tertiary hydrogen bonds are believed to be among the most sensitive to changes in temperature, pH, ionic strength and concentration of polyvalent cations¹⁴, they are likely candidates for modulating flexibility. Breaking these bonds requires 22 kcal mol^{-1} .

After a preliminary examination of various bending motions about several prospective points, we concentrated on the softest bending modes (Fig. 1). The molecule swivels about the region near P8 and P49, with bases 1–7 and 49–76 comprising the upper arm and bases 8–48 comprising the lower. As movement arises from the distortion of several bond angles and dihedrals and the energy minimization algorithm relaxes those distortions by moving several neighbouring atoms, the swivel is not truly a discrete point. Two macroscopic degrees of freedom have been examined, corresponding to the angles α (true bending, with the upper arm rotating about the z axis relative to the fixed lower

arm) and β (swinging, with the upper arm rotating about the y axis while keeping at a right angle to the lower arm). In this study, these two degrees of freedom have been examined separately; a detailed analysis of motions which combine bending and swinging will be reported elsewhere.

Figures 2 and 3 show the results, giving the calculated potential energies for bending and swinging respectively. The most surprising result is that the molecule swivels over an appreciable range of angles using relatively little energy: for $<3 \text{ kcal mol}^{-1}$ it covers a range of about 15° in each direction, while over 30° is covered for $<20 \text{ kcal mol}^{-1}$.

Because these energies represent less than that contained in 10 hydrogen bonds, a number which could easily be made or broken in interactions either with the solvent or with other macromolecules, such interactions clearly could modulate both the overall shape and flexibility of the molecule. While these results do not prove that tRNA^{Phe} is flexible (especially as the solvent is not explicitly treated in the model), they do suggest that there are no large steric or electrostatic forces within the molecule which oppose swivelling. Consequently, we consider it quite likely that tRNAs sometimes have conformations quite different from the familiar L shape and that flexibility can be induced by moderate changes in solvent conditions.

Also interesting is the anisotropy of the forces which oppose the swivelling. The strongest forces (the steepest slopes in Figs 2, 3) are those resisting closing ($\alpha < 0$) and swinging in one direction ($\beta > 0$); they arise from the fact that these two motions pull apart the stack of bases G19–G57–G18–m¹A58 that stabilizes the interaction between the D and T ψ C loops, whereas motions in the other directions ($\alpha > 0$ and $\beta < 0$) maintain that stacking. The $35\text{--}55 \text{ kcal mol}^{-1}$ required to break the stack of bases is an upper limit, as our steepest-descent minimization algorithm probably does not fold the loops into their lowest-energy conformations as they are separated, but it is approximately what would be expected for a stack of four purines, with base stacking interactions of $5\text{--}15 \text{ kcal per mol per stacked pair}$ ^{15,16}.

During the closing motion, the energy drops below $\alpha < -18^\circ$, as the stacks G19–G18 and G57–m¹A58 are formed and as favourable nonbonded interactions are obtained in the inside of the elbow, where many atoms are brought together by the motion. As a more careful folding of the molecule during bending might substantially reduce the height of the energy barrier in Fig. 2, our results give a conservative estimate of the intrinsic flexibility of tRNA^{Phe}, and the molecule may bend even more easily than indicated here.

The flexibility revealed by this study is consistent with the thermal motion observed by crystallographers^{7,17}, who reported that atoms near the ends of the arms move more than those near the elbow. The specific biological functions of this flexibility remain to be established, but these motions may facilitate interactions between tRNA and other macromolecules, and tRNA may assume different conformations at different steps in protein synthesis.

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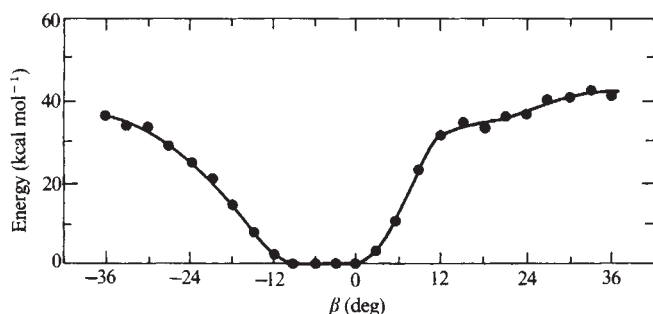


Fig. 3 Dependence of energy on swing angle β , with $\alpha = 0^\circ$.

MATTERS ARISING

Importance of cardiac cell membranes in vanadate-induced NADH oxidation

WE have reported previously¹ that purified cardiac cell membranes with high ($\text{Na}^+ + \text{K}^+$)ATPase activity have significant NADH-vanadate reductase activity. In our experiments, we used calf cardiac cell membranes prepared as described elsewhere², but similar results were obtained with cell membranes from cat and human ventricles and from human erythrocytes. Increasing concentrations of Na_3VO_4 (0.01–1 mM) in the presence of 10 mM imidazole/HCl buffer pH 7.25, 1.25 mM NADH and 0.1–0.2 mg cell membrane protein caused a concentration-dependent oxidation of NADH (followed photometrically by continuous recording at A_{366}), which was virtually absent when heat-denatured cell membranes were used. A more detailed analysis of these experiments has been published elsewhere³.

Recently, Vyskočil and co-workers⁴ reported that vanadate-induced oxidation of NADH does not require a specific enzyme. Moreover, they did not observe a vanadate-dependent oxidation of NADH in the presence of membrane fractions from rat heart and other tissues (skeletal muscle, brain and liver). They found that NADH oxidation in the presence of vanadate proceeded without cardiac cell membranes in the presence of several buffers, except imidazole/HCl, in which the reaction was completely inhibited. Although so far we agree with these results of Vyskočil *et al.*⁴, we tested the vanadate–NADH reaction using rat heart cell membranes and again found vanadate-dependent oxidation of NADH in the presence of membrane fractions. Thus we maintain that cardiac cell membranes (also from rat) contain a heat-sensitive NADH-oxidoreductase that is stimulated by vanadate in the presence of 10 mM imidazole/HCl. Several authors^{5–7} have found a NADH-oxidizing enzyme in plasma membranes of rat liver, rat skeletal muscle and other organs of various species.

A possible reason for the failure of Vyskočil *et al.* to detect the vanadate-dependent oxidation of NADH in the presence of membrane fractions from rat heart might be the procedure used to isolate the membranes or the preparation of the vanadate solution. We used a solution of Na_3VO_4 that was freshly prepared on each day of the experiment in twice distilled water and adjusted to pH 7.25 with HCl.

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VYSKOČIL *ET AL.* REPLY—The following remarks are pertinent to the discrepancies between our experimental data¹ and those of Erdmann *et al.*².

First, the preparation of the vanadate solutions may well have caused some of the differences. In previous papers², Erdmann *et al.* did not describe the preparation of the vanadate stock solutions; other papers^{3–6} have shown that the degree of polymerization of vanadate species depends on the pH and concentration of vanadate in the solution, and on temperature and ionic strength. Particularly at vanadate concentrations of 0.1–10 mM and within the pH range 7.0–8.0, it is uncertain which species predominates, as dimeric, trimeric and decameric clusters may be formed. The possibility cannot be excluded that Erdmann *et al.* worked with vanadate solutions in which decameric anions prevailed, even when freshly prepared. However, we observed no effect in imidazole solution when a stock solution of vanadate (V) was made with NaOH⁷. On the other hand, in the decavanadate solution prepared according to Choate and Mansour⁴, we also observed vanadate-dependent oxidation of NADH in the presence of rat skeletal muscle membrane fractions in an imidazole buffer. It is possible (see ref. 6) that membrane fractions promote the NADH–vanadate (V) reactions by preferential binding of reduced vanadyl (IV), which is consistent with Robinson's statement⁶ that if free ligand(s)—probably some thermolabile components of membranes—are present in a pH-buffered solution, then vanadate (V) ions will tend to be reduced to vanadyl (IV) forms (which bind to the ligand more strongly than the V form) provided there is some electron-donating reagent such as NADH.

Moreover, in an imidazole-buffered medium, most vanadate (V) can be chelated preferentially with imidazole rather than with NADH. In this case, the NADH–vanadate redox reaction (which

proceeds in other buffers within the NADH–vanadate complex^{1,7}) is stopped and needs to be triggered by other ligands (for example, by flavines, F.V., H.D. and J.T., unpublished results).

Another possible source of discrepancy between our results and those of Erdmann *et al.* may have resulted from different treatment of membrane fractions with sodium iodide⁸. Other facts must be taken into account, such as the stability of the preparation² and the possibility of contamination of the cytoplasmic dehydrogenases, which can be attached to the membrane⁸.

For these reasons we believe that within the cell, the oxidation of NADH by vanadate may proceed by forming a complex without any additional support from a specific enzyme, but we do not doubt the validity of the data obtained by Erdmann *et al.* in their specific experimental conditions.

Note added in proof: The rapid non-enzymatic oxidation of NADH (10^{-3} M) by vanadate (10^{-3} M) in phosphate buffer (pH 7.0) is accompanied by the appearance of equivalent amounts of vanadyl(IV) as revealed by the ESR method (Pilař, Vyskočil, Dlouhá and Teisinger, in preparation).

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Matters Arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 500 words and the briefest of replies, to the effect that a point is taken, should be considered.

Lower Palaeozoic oceans and Wilson cycles

ANDERTON¹ has recently suggested that Iapetus (the ocean separating north-west and south-east Britain in the Lower Palaeozoic) may have opened as late as the beginning of the Cambrian. There seems to be a general consensus now that these two parts of Britain were brought together in late Silurian-early Devonian times and that an ocean between them—Iapetus—was thereby destroyed. It has been almost tacitly assumed since the early models of Wilson² and Dewey³ that this ocean must have opened some time before this. Most models (including my own⁴) have assumed that an ocean existed between north-west and south-east Britain for a considerable period. But, from palaeomagnetic reconstructions of the late Precambrian and early Palaeozoic^{5,6}, Iapetus was probably not a two-sided ocean comparable with the Atlantic and there therefore seems little justification for trying to narrow down the timing of opening of a non-existent ocean.

There are few reliable constraints on the relative latitudinal positions of the two sides of the Caledonides in late Precambrian times. One interesting palaeomagnetic result suggests that the Baltic Shield may have lain to the south-east of Scotland in late Grenville times⁷. This position would make the similarities between the Scottish Dalradian and the northern Norway late Precambrian more easily understandable and the 'opening' suggested by Anderton¹ may be allied to the separation of the Baltic and Laurentian shields. This would be regarded as the 'opening of Iapetus' by Scandinavian geologists but is a different concept from that generally envisaged in Britain and it must be remembered that the original designation of Iapetus⁸ was for the Svalbard-Scandinavia-Greenland segment of the Caledonides. Faunal differences between south-east Britain and Scotland thus become irrelevant to the opening of that ocean.

The position of south-east Britain in early late Precambrian and early Palaeozoic times is essentially unknown, but the combination of a distinctly different Precambrian structural history between the two sides and the widely different Lower Cambrian faunas suggests that north-west and south-east Britain were not in juxtaposition at any time before the final closure of Iapetus. Palaeogeographical models^{5,6} have all put south-east Britain at the edge of completely different continental masses from Scotland—and not on the 'other' side of a linear ocean. It is only by rotations and large scale lateral translations (along major transforms of the ocean floor⁹) that the two sides eventually came together at the end of the Silurian. The

Iapetus Ocean thus only existed as a two-sided closing ocean for probably the last part of Ordovician and Silurian times, at least in the Britain-Appalachia segment of the Caledonides.

Many reconstructions suggest completely different patterns of movement for the various sections of the south-east margin of the Caledonian orogeny. Morocco, Avalon (Newfoundland)/New England, Iberia/Brittany, England/Wales, central Europe and Scandinavia may all have moved independently from widely separated positions on the globe to their final resting place against the more or less uniform north-west margin of Greenland-Scotland-northwest Newfoundland-Appalachia. This is fairly unlikely in that one might not expect several microcontinental fragments to collide at even approximately the same time to form the Caledonide orogeny (even allowing for the now well recognized differences in time of collision between Scandinavia and north-west England⁹). There is no evidence that these microcontinental fragments show any evidence of collision with each other before the final closure of Iapetus. The fact that Morocco, Avalon/New England, south-east Britain and Brittany/Iberia also show evidence of a similar late Precambrian history at the margin of a continent suggests that these fragments may have lain along one continental margin¹⁰ which gave rise to the Cadomian cordilleran-type continental margin orogeny.

The sediments of the central European area of this age are marine, probably not shelf sediments, and include a considerable proportion of submarine volcanics, but again the geotectonic nature of the volcanicity and its significance are uncertain. However, it still seems likely that a late Precambrian-early Palaeozoic ocean margin lay along the line of the central European Palaeozoic massifs.

Thus the closure of Iapetus at the end of the Lower Palaeozoic seems a good example of the end of a Wilson cycle. But the late Precambrian and Cambrian history of the two sides of the closing ocean suggests that the opening phase of the Wilson cycle—the splitting apart of the continental mass—probably only affected Scandinavia and Laurentia. In this respect the suggestion¹¹ that the 560-Myr alkaline volcanic¹² and rift system of the North Atlantic may be an indication of the initial splitting may be correct. These volcanics and graben structures occur in Canada, South Greenland and near Oslo on the Baltic Shield, while the Carn Chinné granite of the Scottish Moine area, which is also an alkaline pluton¹³ of this age¹⁴, may well be a Scottish equivalent of this suite. However, the suggestion that the Charnwood Forest volcanics also belong to this event, and even that it lies in a rift structure⁹, are not based on any real evidence. In fact all the volcanics on the south-east side which

have been suggested as remnants of the initial splitting probably belong to a volcanic arc system resultant from subduction under this continental margin⁹.

The whole concept of a Wilson cycle—of continental splitting, ocean widening and continental closure—thus takes on a new light. Study of the closing oceans of many areas through geological time, now made possible by the palaeomagnetic and palaeogeographical compilations of Smith *et al.*⁵ and Zeigler *et al.*⁵, reveals that many collisions are not between continental fragments that had previously been even approximately adjacent, although neither goes far enough back into the Precambrian to describe the early portions of the two sides of Iapetus.

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ANDERTON REPLIES—I agree with Wright about the need to reconsider the Wilson cycle and most of his points about the plate tectonic evolution of the British Isles. As I have discussed the opening of Iapetus in some detail elsewhere¹, I shall just summarize my present views.

I agree that during the Precambrian, north-west and south-east Britain evolved in isolation from each other and that the late Silurian/early Devonian continental collision brought them together for the first time. However, the similarities between Laurentia and Baltica suggest that these areas were part of the same plate during the late Precambrian (although not necessarily in their post-Caledonian relative positions), that this plate split at the end of the Precambrian or in early Cambrian times to form the Iapetus Ocean and that the ocean subsequently closed. Thus, although it does not make sense to talk about the opening of Iapetus in the restricted

context of the British Isles, it may well make sense in a broader, North Atlantic context. However, as Wright implies, the suggested opening and closing of Iapetus between Laurentia and Baltica may well be the exception in plate tectonic behaviour, and the collision of previously unrelated plates, as exemplified by the British Isles, be the rule.

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Spleen colony-forming cell as common precursor of tissue mast cells and granulocytes

THE demonstration by Kitamura and his colleagues¹ of the origin of mast cells from CFU-S is persuasive but to us incomplete. Recipient W/W^v mice with great paucity of mast cells and CFU-S, nevertheless have stem cells which support haematopoiesis and rescue lethally irradiated mice^{2,3}. Donated hemi-syngeneic stem cells from single splenic colonies of normal and beige mice must be few, on average of the order of 10 (ref. 4); only a minority of colonies from the tail of the skew distribution have 100 or more which may be necessary to father without mitotic exhaustion⁵ a viable population of donated haematopoietic cells so as to compete with the host's established haematopoiesis. Kitamura *et al.* claim 23/106 such occasions (Table 1 of ref. 1); but their Table 2 identifies only 2/106 where erythropoiesis was demonstrable in addition to a raised population of 'mast' cells.

Indeed we question whether mast cells have been demonstrated at all. They used as their criterion cells stained with toluidine blue. The 23 reported cases with whole body distribution are highly biased by cells in spleen. It could be that these are basophil granulocytes of myeloid tissue, possibly but not necessarily equated with tissue mast cells. Note that none of the 106 animals showed mast cells in skin, which is repopulated by donor mast cells when compound W mice are repopulated with a generous supply of normal stem cells (ref. 6 and our unpublished results).

Others⁷ have drawn distinctions not mentioned here between skin and intestinal mast cell populations. Is it not likely that cells staining with toluidine blue are quite heterogeneous and that Kitamura *et al.*¹ are observing the effects

of their procedures on just one or a few of these variants?

Our own unpublished observations indicate that all of a variety of tested W compound mice lack cells staining with toluidine blue in both skin and intestine. The mice tested include $W^{sh-2}W^{sh-2}$ (ref. 8), which apart from being black-eyed whites are otherwise substantially normal. Thus the W locus seems to control 'toluidine-blue-affin' cells as well as pigment migration.

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KITAMURA AND YOKOYAMA
REPLY—We have reported that the spleen colony-forming cell is a common precursor of tissue mast cells and neutrophil granulocytes¹. We used mice of two mutant genotypes in the experiment. Giant granules of bg'/bg' mice were used for identification of the origin of both tissue mast cells and neutrophils, and W/W^v mice were useful recipients because they lack tissue mast cells.

Loutit *et al.* have pointed out a discrepancy between the proportion of mice in which mast cells appeared and that of mice in which donor-type erythropoiesis was demonstrable. As they mentioned, W/W^v mice have haematopoietic stem cells but their function is apparently abnormal. Numbers of neutrophils, erythrocytes and tissue mast cells in adult W/W^v mice are respectively ~100, 50 and 1% of the value in congenic $+/+$ mice^{2,3}. The mechanisms which determine such differentiation patterns of abnormal stem cells in W/W^v mice are unclear. However, the haematological situation in W/W^v hosts may explain the discrepancy discussed by Loutit *et al.* The transplanted normal stem cells might be selectively committed to tissue mast cells rather than to neutrophils or erythrocytes.

We think that the 'toluidine-blue-affin' cells counted in our experiments are true mast cells for the following reasons. (1) The cells were not only stained with toluidine blue but also contained

histamine and heparin. The histamine concentration in the forestomach⁴ and the skin⁵ of W/W^v mice is ~1% the value observed in $+/+$ mice. Heparin is not demonstrable in the skin of W/W^v mice⁶. (2) The toluidine-blue-affin cells do not seem to be basophil leukocytes on the basis of their morphology and mode of differentiation. Typical mast cells can be differentiated from typical basophils by morphological criteria⁷. Most toluidine-blue-affin cells which appeared in the spleen of W/W^v mice after transplantation of spleen colony-forming cells were typical tissue mast cells. Moreover, no typical basophils were detected in smears of the bone marrow, spleen or peripheral blood of mice of any genotypes. Basophils, as well as neutrophils and eosinophils, seem to leave haematopoietic tissues after maturation. In contrast, precursors of our toluidine-blue-affin cells proliferated in connective tissues before final differentiation is characteristic of tissue mast cells^{8,9}.

The fact that there was no increase in mast cell number detected in the skin of W/W^v mice after injection of cells from a single spleen colony¹ may be attributable to the differential demands of mast cell differentiation between tissues. Donor-type mast cells appeared in the caecum and stomach but not in the skin when bone marrow cells of bg'/bg' mice were injected into irradiated $+/+$ mice¹⁰. However, the origin of the intestinal mast cells was not different from that of skin because donor-type mast cells also appeared in the skin after local applications of methylcholanthrene to such radiation chimaeras⁹. Moreover, mast cells appeared in the caecum and stomach of 5/9 W/W^v mice but in the skin of only 1/9 W/W^v mice after transplantation of a small number (10⁴) of bone marrow cells from the $+/+$ donors¹¹, this mouse was also cured of anaemia¹¹.

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BOOK REVIEWS

I and brain

Donald Michie

CONCERNING ways in which language limits perception, Paul Henle has observed:

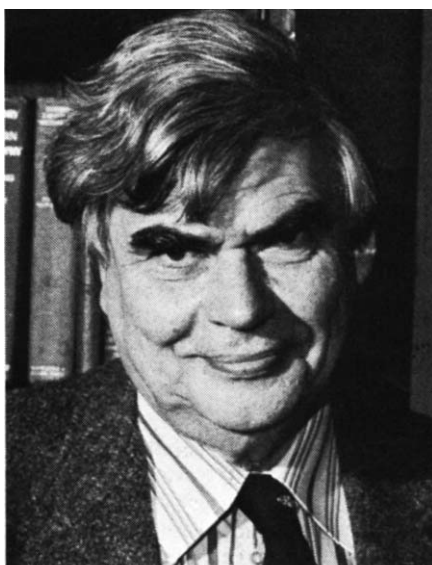
It would seem then to be consistent with what we know of mental set on other grounds to assume that the world appears different to a person using one vocabulary than it would to a person using another. . . . The Navaho, for example, possess colour terms corresponding roughly to our 'white', 'red', and 'yellow' but none which are equivalent to our 'black', 'grey', 'brown', 'blue', and 'green'. They have two terms corresponding to 'black', one denoting the black of darkness, the other the black of objects such as coal. . . . As far as vocabulary is concerned they divide the spectrum into segments different from ours.

The passage is quoted in a new book by our most prominent student of the physiology and psychology of perception, who moreover brings to it an unusual insight into the science of mechanism: Richard Gregory is also a competent and highly inventive engineer.

A decade or more ago his *Eye and Brain* confirmed for perception what Martin Gardner's *Logic Machines and Diagrams* had earlier shown for deductive thought — first, that analysis of brain-mediated operations prospers better on a machine-oriented than a metaphysical base; and second that brilliant popularization is possible without loss of rigour. The intellectual vocabularies which Gregory brought to bear in *Eye and Brain* were those of neurobiology and engineering physics. Both are native to him as a scientist, and they were precisely the two which the task demanded. The new enterprise, *Mind in Science*, far out-demands the old, being no less than a survey from earliest times of the effects of technology upon explanatory theories in science.

The requirement for a multi-lingual intellect imposed by such a task seems awe-inspiring. The book's 600 pages sweep through the origins of myths and of tools, the invention and nature of writing, of mathematics and of machines; through natural philosophy from pre-Socratic to post-Einsteinian; through early notions of biology and medicine to the origin of species and to the descent and cultural ascent of man; to the measurement and definition of intelligence, the philosophy of mind, the physiology of brain, the psychology and metaphysics of perception, and finally to language, consciousness and the nature of knowledge and truth. Interwoven through the narrative are strands of sociology, mechanics, thermodynamics, cybernetics (this last is genially labelled by Gregory's broad brush as "Artificial

Mind in Science: A History of Explanations in Psychology and Physics. By Richard L. Gregory. Pp.641. ISBN UK 0-297-77825-0; ISBN US 0-521-24307-6. (Weidenfeld & Nicolson/Cambridge University Press: 1981.) £18.50, \$29.95.



Richard Gregory — a good companion

Intelligence") and of symbolic logic.

Equally breathtaking is how close Gregory comes to success. It is as though some Leonardo of the Navaho had essayed a treatise on pigments of many lands, and had almost accomplished the heroic act while still, to return to Henle, having but a single term for "grey" and "brown", and another for "blue" and "green". The mental vocabulary missing from Gregory's near-masterwork is that of computation, interpreted broadly to include the principles of modern predicate logic and the means of implementation adopted by the designers of the new generation of intelligent computing systems. In the result, whenever we follow the author to the active modelling of thought by machine, our thirst intensifies; but it is at these very points that the stream dries.

The abstract forerunner of the modern computer is A.M. Turing's startling and consequential theoretical construct, the Universal Turing Machine. One might expect this historic intellectual mechanism to figure prominently in a book with such a theme as Gregory's. It comes as a surprise to find it boiled down to " . . . the important notion advanced by the pioneer computer scientist at Manchester, Alan

Turing: that anything that is conceivable can be represented by a simple machine, capable of a very long string of alternative states". The "important notion" was in fact advanced in the mid-1930s when Turing was at Cambridge. When he moved at Max Newman's invitation to Manchester in September 1948 it was *not* to "found the Manchester computing laboratory" as stated later in this book (p.382). By that time Tom Kilburn and F.C. Williams had already achieved operation of the Manchester prototype on which they had embarked in December 1946. But more serious is the author's failure to grasp, and hence to communicate, what the whole Babbage-Turing-Kilburn-Williams-Wilkes-Eckert-Mauchly-von Neumann story was actually about.

Richard Gregory has one of the most richly furnished and dazzling minds that I have ever encountered. But for every person, there are one or two subjects which run so widdershins to the natural grain as to become subtly repellent, almost "anti-subjects". For myself, however long and intently I strive, I cannot learn a foreign language. Gregory cannot learn a computer language. At Edinburgh he was briefly enticed into the attempt. But the terminology of tests and loops seemed to grate so painfully that the experiment was dropped. Here is a clue to what can make a scholar attached to historicity and precision go awry. When the anti-subject returns to mind, repellency is re-awakened and the instruments of memory are jarred. Thus:

I was captured by the dream of AI while it was yet a seed without flower or fruit: to leave Cambridge to help my colleagues Donald Michie and Christopher Longuet-Higgins to set up the Department of Machine Intelligence and Perception at Edinburgh University. Here we collaborated with the corresponding Departments at MIT and Stanford, and we built the Robot Freddy, which was one of the first to recognise objects from any point of view and to make models.

A meccano mounting of a TV camera on two small motor-driven wheels had indeed been used by Barrow and Popplestone (see their subsequent 1971 paper in *Machine Intelligence* 6) for their own preliminary skirmish with the vision problem before Gregory's departure to Bristol in September 1970. But the robot to which he must surely be referring was then still at the planning stage, to be completed, and its computer-based accomplishments demon-

strated, 2½ years later. The team which saw the Freddy project through consisted of A.P. Ambler, H.G. Barrow, C.M. Brown, R.M. Burstall and R.J. Popplestone (see their paper in *Artificial Intelligence* Summer 1975); and on the hardware side S. Salter (mechanical engineering) and Gregan Crawford (electronics).

It should also be recalled that the founding giants of AI, men such as Samuel, Simon, Newell, McCarthy, Minsky, Robinson and Rosen had been labouring to set the cornerstones some ten years before Gregory was captured by his dream of flower and fruit. But the bold literary approximation sometimes economically conveys a deeper truth. Before he left Edinburgh Gregory taught us that if a robot is to see, then its programmers must enable it to *reconstruct* its percepts, as does the human being, from a stored inventory of how the components of familiar objects and scenes normally look. This Helmholtzian doctrine is a cliché of computer vision today. In those days it was not, and the launching orientation was derived, to speak for myself, in powerful measure from Gregory.

From time to time in the book this gift flashes forth to astonish, illuminate and (occasionally) to infuriate. It is human to be irked by an inspired guide who against overwhelming odds brings one to the right door, only to signal by tell-tale fumbings that he lacks the door key. But is it rational to be irked? Who else could have piloted one thus far? Moreover one is perfectly free to thank him and look for a key-owner or a locksmith. Gregory has seen from afar that the instruments of thought-automation are the "tools", "materials" and "processes" of a new industry, the technology of human knowledge. On approaching closer, his expertise gives out, just where that of the new race of knowledge engineers begins. The important thing, however, is that his insight is correct.

The book's pivotal chapter, "The Nature and Nurture of Intelligence", will stir profitable thought in laboratories where man-machine synthesis of prototype knowledge systems is making precise what Gregory's mind's eye has spied. As with the book as a whole, the chapter tells everything about the philosophy of AI, nothing of the method.

It also tells on every page a very great deal about its author. From this comes many flaws, but much charm. One of the joys of the scientific life is to drop for a while the customary pendants in exchange for good companionship. *Mind in Science* offers sustenance of just this kind. Possibly some scientific or philosophical people may abstain from its pages. They must be warned that they will have to continue their journey more poorly supplied than need have been the case. □

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Cell transformation: a missed opportunity

Daniel Rifkin

Neoplastic and Normal Cells in Culture. Developmental and Cell Biology, 8. By J.M. Vasiliev and I.M. Gelfand. Pp.372. ISBN 0-521-23149-3. (Cambridge University Press: 1981.) £40, \$79.50.

THE enormous increase of interest over the past ten years in the molecular biology of tumour viruses, as well as in the physiology of cultured cells, has created an extensive literature on the properties of normal and transformed cells, and on the relationships of these properties to the cellular phenotype. There is no book currently available which brings together the large body of information accumulated in this area. Thus, it was with pleasure that I began Vasiliev and Gelfand's book *Neoplastic and Normal Cells in Culture*. The aim of the authors is "to describe and discuss comparative characteristics of the interactions of normal and neoplastic cells with their environment in cell cultures". The book, then, would appear to be the perfect vehicle for a synthesis of data on those properties of neoplastic cells responsible for their altered growth, their invasive behaviour and their metastatic potential, specifically as measured by *in vitro* criteria.

The book is divided into four parts. Part 1, an introduction, is subdivided into two sections: the first covers the general properties of neoplastic cells, especially growth, and the second deals with neoplastic transformation in culture by a variety of agents. The second part of the book, "Morphogenesis in 'Normal and Transformed Cultures'", is concerned with cellular structures and their function in relation to morphogenesis. This includes the movement of normal fibroblasts and epithelial cells in relation to their morphology, the locomotion and morphology of tumour cells, and specific biochemical or cytological processes whose alteration may be responsible for the difference between normal and transformed cells. Part 3 deals with growth regulation in normal and transformed cells, concentrating on the anchorage, density and soluble factor controls of cell division, and the interactions of these phenomena. The book concludes with a general discussion based on a comparison of *in vitro* and *in vivo* malignant cells as well as the interrelationships of transformation properties.

Within the scope of the authors' aims, the book is filled with interesting references and intriguing observations. The authors present a number of thoughtful conclusions and certainly point out certain ambiguities in the data from different laboratories.

However, the book has two serious deficiencies. The first of these is the cursory discussion of the possible significance of

cell-matrix interactions in determining the cellular phenotype. The possible influences of fibronectin, collagen and proteoglycans on cell behaviour are given little more than two paragraphs. This is a rather exciting area of current experimentation but it is presented essentially as an adjunct to the more morphological research. This, in fact, is the second shortcoming of the book — the emphasis on morphological aspects to the virtual exclusion of biochemical analysis. An enormous variety of cellular phenomena is described, but only in a few instances is there any discussion of the possible molecular basis for these phenomena.

But perhaps the most disappointing aspect of the book is its blandness. All of the information presented receives much the same degree of emphasis and enthusiasm. The authors rarely indicate a preference for one interpretation versus another, nor do they ever criticize omissions in the data, the lack of proper controls or internal inconsistencies. This is not a critical presentation of the data. It was rather disheartening to read through a book concerned with an area of such excitement and find only a compendium of facts. The failure to create any larger order out of the enormous amount of information available must be counted as a missed opportunity. □

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Resources on a plate

Frederick J. Sawkins

Economic Geology and Geotectonics. Edited by D.H. Tarling. Pp.213. ISBN UK 0-632-00738-9/ISBN US 0-470-27145-0. (Blackwell Scientific/Halsted Press: 1981.) £15, \$54.95.

THE possible relationships of resources of various kinds to geotectonics is a subject that is attracting considerable attention nowadays, and this volume was apparently conceived with the object of providing an introduction to anyone — undergraduates, postgraduates and even seasoned researchers — with an interest in the field. Sadly, the book failed to live up to my initial expectations. It contains nine chapters by eight contributing authors, and opens with an introduction to the general theory and operation of plate tectonics. Although somewhat sparsely illustrated, this account provides a competent

overview and contains a substantial bibliography. One or two comments were rather surprising, however, especially the likening of greenstone belts to Mesozoic continental flood basalts.

The following three chapters deal with the formation, migration and accumulation of petroleum. Much of the material is a duplication of that to be found in any petroleum geology text. Plate tectonics is certainly dealt with in terms of basin development, but in view of the volume's title and its stated goals, this important subject deserved a more penetrating analysis and further fleshing-out with specific examples. Chapter 5 provides the reader with an overview of coal geology; again, much of the material is standard textbook stuff, and the chapter contains only half a page of general comments regarding possible plate tectonic controls of coal-generating environments.

The remainder of the book covers the relationships of metal deposits to plate tectonics. The chapter dealing with metal deposits formed at spreading ridges is well done and covers podiform chromite and Cyprus-type massive sulphide deposits. However, the association of ore deposits with subduction-related environments is covered in a mere seven pages of text, plus four illustrations; as a result of this brevity, many important types of deposits in subduction-related arcs are ignored, as are most of the more recent insights into arc-metal deposit relationships such as inter-arc rifting.

The contribution on the origins of ore deposits in sedimentary rocks is certainly of adequate length and contains a good deal of information, although the writing is somewhat laboured. I was also confused by the author's lack of conviction on certain points. For example, less than a page after a statement that the relationship between "secondary sedimentary ores and some sort of growth faulting is so common as to be almost a requirement", the suggestion that rifting is active during mineralization is questioned.

The final chapter considers the significance of palaeoclimatic factors on resources and offers some general conclusions. It touches briefly on a host of varied topics, but contains one or two startling misconceptions, for example the suggestion that the copper-sulphide deposits of Kennecott, Alaska, have a detrital origin.

The volume contains 64 figures, many of them rather simple sketches, and only a single table. There are also a number of text citations missing in the references.

Overall, one is left with the impression of a somewhat hastily assembled volume which falls short of its claim to "provide an integrated introduction to the subject of economic geology". ☐

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Solving big problems on big computers

F. James

Computer Simulation Using Particles. By Roger W. Hockney and James W. Eastwood. Pp.540. ISBN 0-07-029108-X. (McGraw-Hill: 1981.) \$49.50, £27.50.

It is a pleasure, in these days of makeshift books on computing and simulation, to come across a thoroughly coherent work painstakingly prepared by recognized experts in the field.

The authors address themselves to one of the three general approaches to the study of systems (liquids, galaxies, plasmas and so on) governed by known differential equations. The other two approaches, finite elements and finite differences, are already well covered in the literature, whereas direct simulation by following constituent "particles" of the system, although in many ways more interesting mathematically, has largely been ignored except in research papers. To say that this book fills the gap is something of an understatement.

The orientation of the book is truly that of computational physics (or, as the authors prefer, computational science). It is not just a physics book with a chapter on FORTRAN at the end. Although the subject is problems arising in physics and related areas, suitability to computation is the overriding criterion for all of the material presented; no model or technique is suggested which has not been used in actual computations, more often than not by the authors themselves.

Considerable attention is paid even to programming methodology, including a rather complete account of the OLYMPUS programming system. Such a chapter could, of course, be grafted onto any book, but it finds a natural place in this work where the coverage of fundamental properties of algorithms includes not only their theoretical convergence rates, stability and such, but also actual computer time and storage requirements, arithmetic precision requirements and sometimes suitability to particular computer architectures. The usefulness of this aspect could have been enhanced still further by including a table of comparison of different computers' performance, since of the computers most often cited (IBM 7090, 360/91 and 360/195, CDC 6600 and 7600, ILLIAC IV, CRAY-1) most are rare birds, technologically obsolescent or both, and, apart from the CRAY, are unlikely to be commonly available in a few years' time.

Physicists will appreciate the authors' basic approach, the mathematical notation

and the vocabulary. The reader is encouraged to consider the problems in their physical context, not just as mathematical abstractions. The importance of physical insight in the choice of mathematical methods is brought out clearly. In addition, the interdisciplinary nature of the work should make it especially interesting, for example, to an astronomer who can see the relationships between problems arising in the study of the orbits of galaxies and in the design of transistors.

The style of the book is characterized by clarity and simplicity, the hallmarks of authors who have something of substance to say and possess a complete grasp of their subject. The quality of the printing, the pleasant layout of text and, especially, the numerous and helpful illustrations (many computer-produced, of course) all contribute towards making the book a real pleasure to read. It is a first-rate reference book which will be indispensable to workers in the field, and it will certainly find its place as a textbook for advanced courses in both computer science and physics, as well as intermediate fields. ☐

Frederick James is a staff physicist in the Data Handling Division of CERN, and a past chairman of the Computational Physics Group of the European Physical Society.



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$P = s \cdot \exp(E/kT)$: common ground in thermal physics

G.F.J. Garlick

Analysis of Thermally Stimulated Processes. By R. Chen and Y. Kirsh. Pp.361. ISBN 0-08-022930-1. (Pergamon: 1981.) \$60, £25.

WHAT might there be in common among such apparently diverse experimental operations as the thermal stimulation of luminescence or current on the one hand, and thermogravimetry or differential thermal analysis on the other? Add to these other processes measured in, for example, thermal annealing, evolved gas analysis, thermoremanent magnetism, thermal desorption, dilatometry and thermally induced creep, and there is the subject matter of the present text.

In their preface, the authors themselves make the comment that the book could look like a "marriage of convenience". However, there is more connection between the effects than that they each involve a change in some measurable physical parameter when a material sample is warmed up. The more subtle factor common to them all is the theoretical expression for the probability of the effect, which is the familiar Arrhenius exponential function $P = s \cdot \exp(E/kT)$, where s is often known as the "frequency constant", E is the energy needed to effect the change in parameter, k is Boltzmann's constant and T the absolute temperature. The major problems of developing theory to correlate with any of them are not about this function but about the kinetics which determine what happens later. For instance, will an escaping electron recombine with its original location to give, for example, luminescence, or will it be retrapped?

A large part of the book is taken up with these kinetic variations and the reader interested in thermogravimetry or differential thermal analysis may find that his interests are somewhat overwhelmed by extensive treatments of thermally stimulated luminescence and conductivity. The volume will certainly not replace the many treatises on thermogravimetry or differential thermal analysis and other specific techniques to which the users of the associated, sophisticated equipment now turn. However, there is always the possibility that by reading of the existence of a wider range of thermally stimulated effects ideas may be transferred between the disciplines. In this sense the text will be very useful.

With the major concern of the authors being thermally stimulated electronic processes in insulators and semiconductors, there is a fairly exhaustive coverage of the theory and practice of studying "deep" levels in such solids. There are, however, some omissions and some inaccuracies. On a historical note, more credit should have been given to

Urbach for his quantitative assessment of thermoluminescence in 1930 which preceded that of Randall and Wilkins by many years. On the matter of retrapping the authors do not, in drawing examples from the literature, bring out the still-puzzling case of zinc sulphide phosphors, where luminescence and conductivity can be co-explored to determine detailed mechanisms. In 1948 Wilkins and I (*Nature* **161**, 565–566) showed that retrapping of thermally released electrons was very small even when empty traps outnumbered empty recombination centres by more than 1000:1. In another instance, when reviewing photon-induced transfer of carriers between trapping states, the enormous effect found in infrared-stimulable zinc sulphides by myself and Mason (*J. Electrochem. Soc.* **96**, 90–113; 1949) is not referred to. These are important examples because they indicate ways in which retrapping can be examined in an important group of high band gap solids.

In spite of the problems of using thermally stimulated processes to obtain

accurate values of trap energies and cross-sections, the recent development of DLTS (deep level transient spectroscopy) as a steady state method of thermal stimulation would appear to offer a breakthrough for determination of these parameters in device semiconductors such as silicon and gallium arsenide. It is a pity that some of the elegant examples of trap distributions obtained by this method were not included in the text.

There is no doubt that this book will be attractive to particular groups of readers who, like myself, have specific interests in carrier trapping and related processes in insulators and semiconductors. It is well referenced and has a critical approach to the use and interpretation of thermal stimulation methods. Whether its attempt to coordinate analysis of the many different thermally stimulated effects will appeal to a wider readership is less sure.

G.F.J. Garlick is a consultant specializing in the fields of luminescence and electronic processes in semiconductors.

Developments in theorists' views of ecology

Richard Law

Theoretical Ecology: Principles and Applications, 2nd Edn. Edited by Robert M. May. Pp.489. ISBN hbk 0-632-00768-0; ISBN pbk 0-632-00762-1. (Blackwell Scientific/Sinauer Associates: 1981.) Hbk £20, \$42.75; pbk £10.50, \$23.95.

THEORETICAL ecology is a fast growing subject due, in large measure, to the stimulus of R.M. May and his associates; it is pleasing, therefore, that they should take the time to produce a new edition of their standard work (first published in 1976).

While retaining the same overall theme of developing a theoretical perspective from which to view the living world, the new edition contains some substantial changes. Several chapters have been modified in the light of recent work — most notably that on patterns in multi-species communities, which now considers intriguing questions such as how many trophic levels should exist and how theories about community stability and complexity can be tested. Several other chapters have been rewritten by new authors; in particular, that on plant-herbivore systems has been improved by taking greater account of herbivory in the real world. However other chapters that would have benefited from revision remain unchanged. For example, "Island Biogeography and the Design of Nature Reserves" hardly does justice to the

promise of its title, design principles for nature reserves being relegated to a few paragraphs at the end. No mention is made of recent work in this area and of the debate that has come to surround the whole subject.

There are two welcome additions to the book. The first is a survey of the new kind of population dynamics that is emerging from studies of plants, in which individuals, rather than growing to a fixed size, may continue to grow indefinitely by the production of modular units of construction. The second, dealing with the economics of biological resources, follows an elegant path from ecological theory to practical questions of optimal harvesting and the regulation of fisheries.

The book does not attempt to give a comprehensive coverage of theoretical ecology — for example, the reader will search in vain for an adequate account of the dynamics of populations with age distributions or of the effects of genetic variability in ecological parameters. But any shortcomings are greatly outweighed by its merits; it is a lucid and up-to-date introduction to the contributions of R.M. May and his associates to theoretical ecology, and it deserves to be widely read by all those with an interest in the subject.

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